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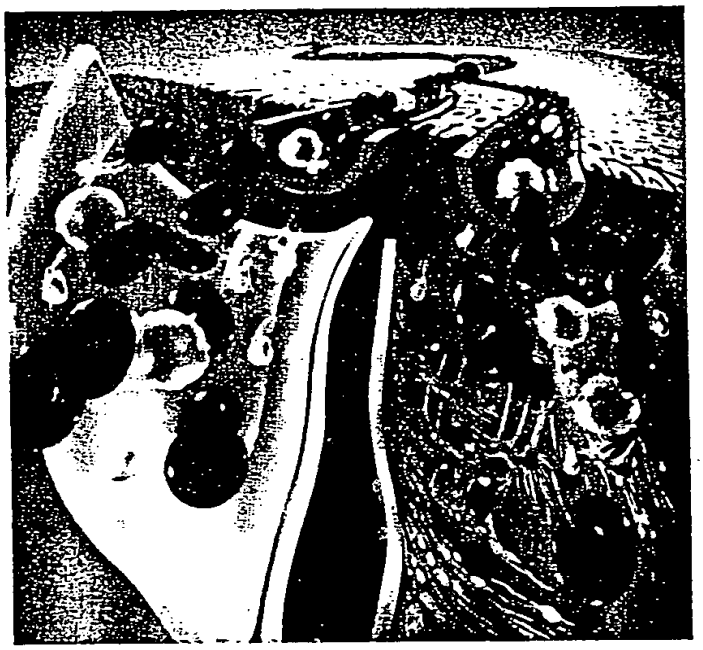
Exhibit C

DURAFLO®

Biocompatible

Treatment



Baxter

Improved Patient Outcomes.

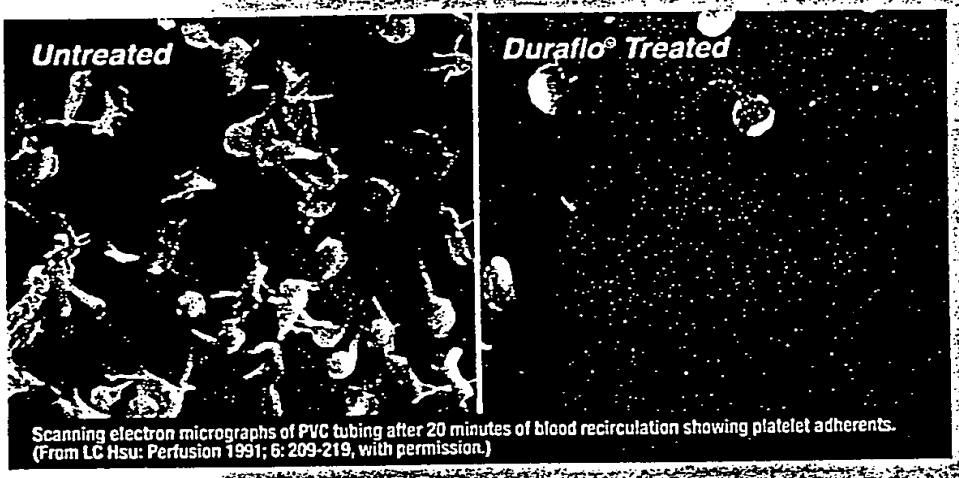
These are the goals of products and technologies that have been developed over the years that are seen today as fundamentally essential. Biocompatibility is a new focal point to further enhance patient outcomes. It is this focus that has led to the development and utilization of the Duraflo® biocompatible treatment.

What Is Biocompatibility?

Biocompatibility is the creation of a surface that alters the unfavorable biological response to a foreign substance.

Why Is Biocompatibility Needed?

Exposure of blood to synthetic surfaces generally leads to activation of coagulation and blood systems. In order to further improve patient outcomes, a paradigm shift is taking place in the medical device industry to more biocompatible product lines, and Baxter Healthcare Corporation is at the leading edge of this innovative trend.



Scanning electron micrographs of PVC tubing after 20 minutes of blood recirculation showing platelet adherents. (From LC Hsu: Perfusion 1991; 6: 209-219, with permission.)

What Is The Duraflo® Biocompatible Treatment?

The Duraflo® biocompatible treatment combines U.S.P. grade heparin with a proprietary binding agent. The combination of the heparin and the binding agent is universally applicable to most surfaces, resulting in a biocompatible surface.

How Does The Duraflo® Biocompatible Treatment Work?

Several studies have shown that the Duraflo® treatment reduces the whole body inflammatory response and potentially improves patient outcomes in two ways. First, the biocompatible treatment alters the normal sequence of plasma adsorption on Duraflo® treated surfaces resulting in reduced fibrinogen adsorption. Fibrinogen adsorption is undesirable because fibrinogen is a precursor to fibrin (clot) and platelets adhere to surfaces containing fibrinogen. The adhered platelets undergo morphological changes and become activated - thereby forming platelet aggregates. With less fibrinogen adsorption Duraflo® treated surfaces reduce platelet activation, platelet adhesion, and platelet depletion.

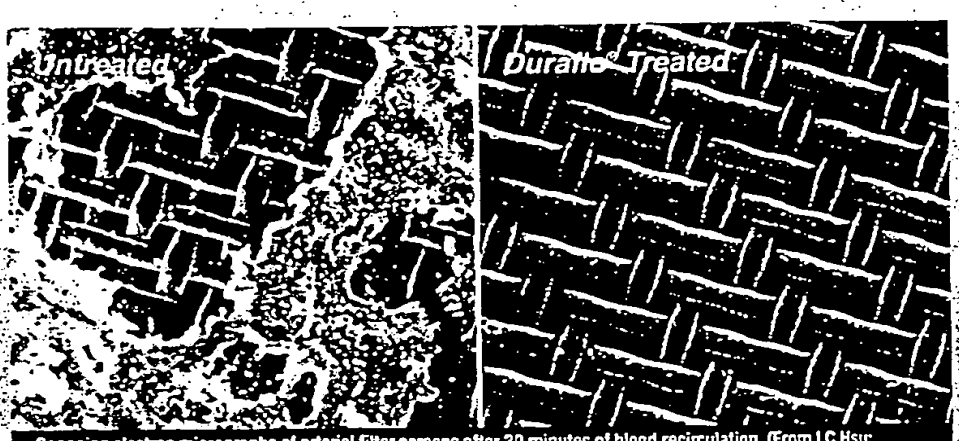
Second, the Duraflo® treatment interacts with anti-thrombin III and may inhibit contact enzymes such as Factor XIIa and kallikrein at the initial point of blood interaction.

The cover depicts blood interacting with Duraflo® treated and untreated surfaces. On the right side, the detrimental effects of the blood's foreign surface interactions are shown, and on the left, the gentle biocompatible nature of the Duraflo® treated surface is illustrated

This preemptive interaction helps limit the activation of the coagulation, complement, and fibrinolytic systems - thus limiting the whole body inflammatory response.

Is The Duraflo® Biocompatible Treatment's Use Limited To Certain Medical Devices?

No. Although the primary applications to date have been in the cardiopulmonary bypass market, several projects are underway to apply the Duraflo® treatment to a wide range of medical devices. The Duraflo® treatment's anti-thrombogenic and anti-inflammatory benefits are applicable in virtually all cases where blood interacts with a foreign material.



Scanning electron micrographs of arterial filter screens after 30 minutes of blood recirculation. (From LC Hsu: Perfusion 1991; 6: 209-219, with permission.)

To What Materials Can The Duraflo® Treatment Be Applied?

The Biocompatible Technologies Group at Baxter has developed and is currently developing expertise with many different types of polymeric and metallic materials. These materials include, but are not limited to the following:

- ABS - (Acrylic-butadiene Styrene)
- Acrylic & Methacrylic Polymers
- Aluminum
- Cellulosic Materials
- Polyamide
- Polycarbonate
- Polyester
- Polyethylene
- Polyisoprene
- Polypropylene
- Polysulfone
- PTFE - (Polytetraflouroethylene)
- Polyurethane
- PVC - (Polyvinyl Chloride) Flexible and Rigid
- Pyrolytic Carbon
- Silicone
- Stainless Steel

For material applications not listed, please contact the Biocompatible Technologies Group.

Is The Duraflo® Biocompatible Treatment Technology Only Being Used On Baxter Devices?

No. Several Duraflo® treatment licensing agreements have already been signed for non-Baxter products. In addition, multiple licensing agreements are presently being negotiated for additional products. Applications of the Duraflo® treatment technology are very broad and expand across many areas of the medical device industry.

What Are The Benefits Of Using The Duraflo® Biocompatible Treatment Over Other Coatings?

The Duraflo® treatment has been established as a world leader in biocompatible coatings. With over 40 published clinical papers demonstrating its benefits, the Duraflo® treatment has strong brand name and market recognition. Within cardiopulmonary bypass, clinical and in-vitro studies have shown substantial reductions in post-operative blood loss and significant reductions in such potentially harmful reactions as platelet activation, platelet depletion, fibrinogen adsorption, thrombus formation and complement activation. The bottom line is improved patient care. Other Duraflo® treatment benefits include:

- Extensive experience gaining FDA and international product clearances on Duraflo® treated products.
- Large scale manufacturing experience and engineering expertise in process application, manufacturing and product qualification.
- Multiple licensing agreements.
- Applications for Duraflo® treated products outside the cardiopulmonary arena underway.
- Broad advertising and marketing to establish the Duraflo® treatment technology as the biocompatible treatment standard.

How May I Learn More About Duraflo®?

Simply contact the Biocompatible Technologies Group of Baxter Healthcare Corporation at 714.756.4364 or 714.756.4500.

COATED FOR DURAFLO® BIOCOMPATIBILITY

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Heparin-Bonded Surfaces in Extracorporeal Membrane Oxygenation for Cardiac Support

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Development of increasingly complex perfusion devices with bonded heparin allowed for significant improvement of thromboresistance of most basic components required for cardiopulmonary bypass. In his recent review of heparin-coated cardiopulmonary bypass circuits, Gravlee cited 91 references dealing with heparin-coated surfaces, and far more can be found if the search includes material technology or heparin-coated devices not designed for cardiopulmonary bypass (eg, ventricular assist devices, hemofilters, catheters). The present review is focused on long-term application of heparin-coated equipment in conjunction with basic work on heparin bonding relevant for extracorporeal membrane oxygenation. Experimental open chest cardiopulmonary bypass using heparin-coated equipment without systemic heparinization up to 36 hours has shown improved thromboresistance, and better platelet preservation was demonstrated for perfusion with heparin-coated cardiopulmonary bypass equipment up to 5 days in the experimental set-up. Similar findings were reported for roller pump perfusion with heparin-coated tubing and centrifugal pump perfusion with heparin-coated pump heads. More recently, heparin bonding was also made available for oxygenators with true membranes that preclude plasma leakage. The available knowledge on clinical applications of heparin-coated perfusion equipment is mainly based on short-term applications like ours, which now includes more than 300 patients. Reduced postoperative blood loss and as a result fewer transfusions were the main benefits of heparin-coated equipment allowing for perfusion with low systemic heparinization. There are only a few reports on long-term use of heparin-coated equipment for prolonged circulatory support. However, the longest clinical application of a single device is that of an intravascular gas exchanger that remained fully functional during a 29-day implantation period. Finally, it appears, that circulating protamine interacts with surface-bound heparin. Protamine administration should therefore be avoided during perfusion with heparin-bonded equipment to maintain the improved thromboresistance.

(Ann Thorac Surg 1996;61:330–5)

Since their first description by Gott and associates in 1963 [1], heparin surface coating techniques for perfusion devices have undergone significant evolution. Not only were chemical modifications made to improve the retention of heparin on a multitude of artificial surfaces, but the antithrombotic activity of the modified surfaces was also increased. Furthermore, the introduction of modern membrane oxygenators with systematic separation of blood and gas flow paths enabled investigators to improve the entire blood-exposed surface of an extracorporeal circuit including an oxygenator.

Over the years, stepwise introduction of more and more complex perfusion devices with heparin surface coating resulted in significant improvement in thromboresistance of most basic devices required for cardiopulmonary bypass [2]. Clinical application of heparin-coated perfusion equipment taking advantage of the improved thromboresistance to reduce systemic heparinization followed the progress made in the experimental set-up, at least for short-term application [3]. In his recent review of heparin-coated cardiopulmonary bypass circuits, Gravlee [4] cites 91 references dealing with heparin-coated surfaces, and far more can be found in the recent literature if the search includes material technology or heparin-coated devices used outside of cardiopulmonary bypass such as ventricular assist devices [5]. However, there are only a few reports on long-term application of heparin-coated equipment, and therefore we will review a number of the latter studies in conjunction with basic work on heparin-coated equipment relevant for extracorporeal membrane oxygenation (ECMO).

Open Chest, Partial Cardiopulmonary Bypass With Heparin-Coated Equipment

After a number of disappointing clinical experiences with long-term cardiopulmonary bypass after cardiotomy using standard equipment with full systemic heparinization, we have evaluated the potential of open chest perfusion with heparin-coated equipment in a canine model [6, 7]. Duraflo II (Baxter-Bentley, Irvine, CA) heparin-coated tubing sets including a heparin-coated flexible venous reservoir, a heparin-coated heat-exchanger hollow-fiber membrane oxygenator structure, and a heparin-coated arterial filter were used. Open chest perfusion (right atrium to aorta) without systemic hepa-

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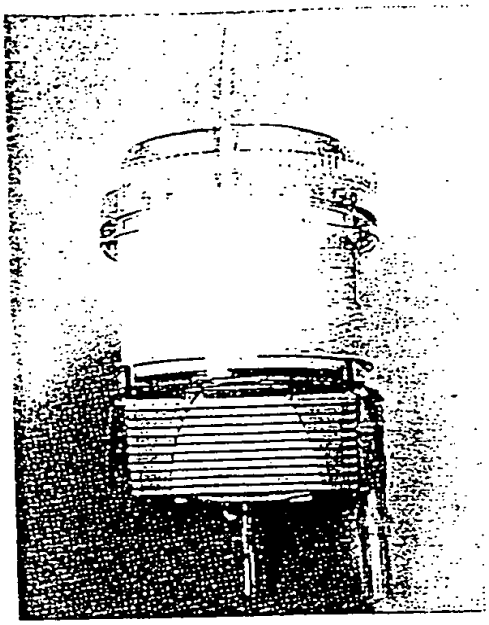

Fig 1. Carmeda Bio-Active System heparin-coated Maxima oxygenator carefully rinsed after venoarterial perfusion in a bovine experiment with low systemic heparinization: only minimal deposits are visible in the heat exchanger section.

improved thromboresistance were documented for the heparin-coated ECMO circuits.

True Membranes With Heparin-Coated Surfaces

The Kolobow silicone rubber membrane lungs (previously marketed under the Scimed brand) have been the gold standard for ECMO over many years. The silicone rubber blood–gas interface is acting as true membrane, which prevents plasma leakage as long as the integrity of the devices is not disturbed. Because of the uneven flow patterns throughout the spiral membrane, however,

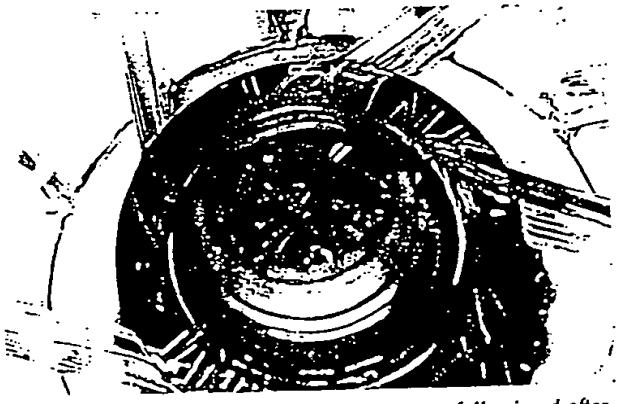

Fig 2. Uncoated Sarns 3M centrifugal pump carefully rinsed after left heart bypass in a bovine experiment without systemic heparinization: challenging bovine experiments result in significant impeller deposits.

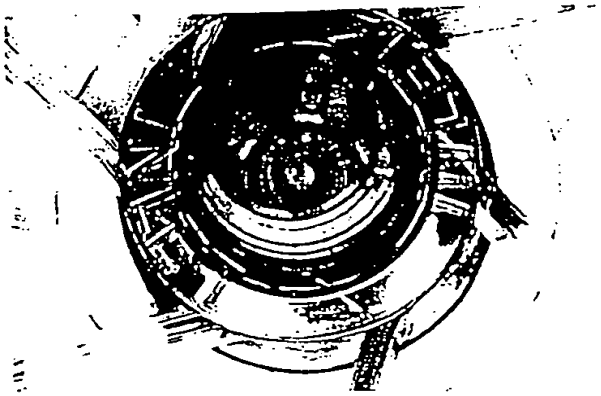

Fig 3. Duraflo II heparin-coated Sarns 3M centrifugal pump after left heart bypass in a bovine experiment without systemic heparinization: clean device.

some degree of coagulation within the devices is not uncommon, especially if low or no systemic heparinization is used. During prolonged ECMO procedures, decreasing gas transfer requiring device exchange has therefore triggered several attempts to produce heparin-coated oxygenators. Figure 4 shows an unrolled membrane segment of a heparin-coated Kolobow oxygenator (Ultrox; Avecor, Plymouth, MN) that was studied in our laboratories after bovine experiments without systemic heparinization. The whole surface, including its borders, is macroscopically clean. Unfortunately, no heparin-coated Kolobov membrane oxygenator has been made available for clinical practice.

A different concept was used in the diffusion oxygenator developed by Jostra Medizintechnik GmbH, Hirrlingen, Germany. This device is built from a pack of microporous plates that are covered by a tight silicone membrane. Hence, plasma leakage is virtually impossible. In bovine studies performed in our laboratories [17]

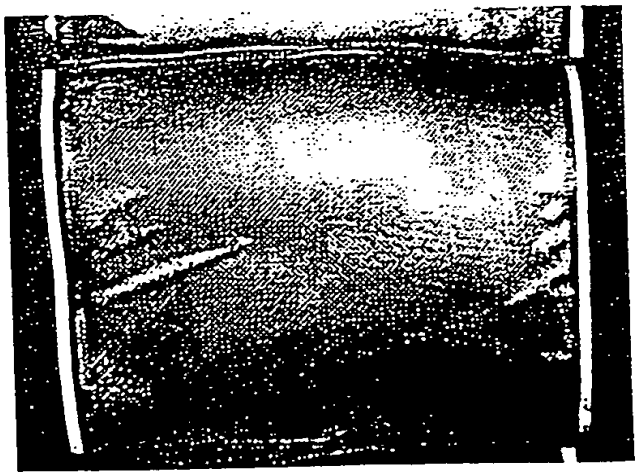

Fig 4. Unrolled segment of a heparin-coated Kolobow membrane oxygenator after venoarterial perfusion in a bovine experiment with low systemic heparinization: borders are free of red clots.

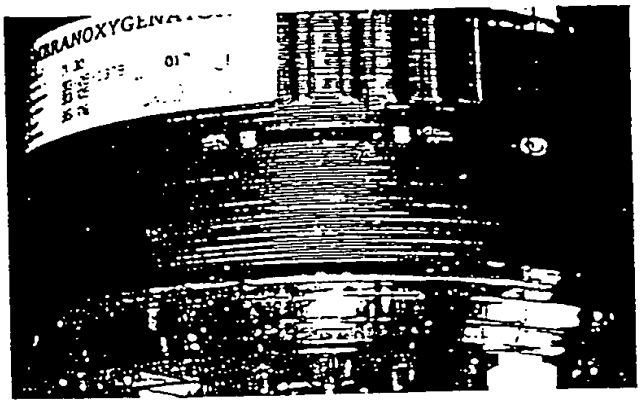

Fig 5. Bioline heparin-coated Jostra silicone plate membrane oxygenator carefully rinsed after venoarterial perfusion in bovine experiments with low systemic heparinization: oxygenating compartment is free of red clots.

the heparin-coated version (Bioline) appeared to have good antithrombotic properties (Fig 5), which have yet to be confirmed in clinical applications.

Clinical Experience With Heparin-Coated Equipment

Clinical application of heparin-coated perfusion equipment for treatment of adult respiratory distress syndrome [18] followed successful shunting procedures with heparin-coated devices in repair of descending aortic aneurysms. There is now an increasing number of reports in the literature addressing ECMO in the management of cardiac failure. Zwischenberger and Cox [19] refer to the ECMO registry of the Extracorporeal Life Support Organization (Thomas Tracy, St. Louis University Medical Center, St. Louis, MO). By 1994, more than 1,000 pediatric ECMO procedures for treatment of refractory cardiogenic shock before or after repair of congenital heart defects with a survival rate of 44% were registered. Hemorrhage was the most common complication. There was also a group of adult patients (40 cases) who underwent ECMO for cardiac support with a 15% survival rate. Again, bleeding was the most common complication. Unfortunately, the data on file do not allow distinguishing between procedures using standard perfusion equipment as compared with heparin-coated equipment.

Klein and colleagues [20] reported on 36 children supported with ECMO after repair of congenital heart disease with a survival rate of 61%. All patients underwent venoarterial ECMO with standard circuits using Kolobov membranes (longest procedure, 215 hours). Bleeding related to heparinization was the most common complication and is probably the most important factor in the move toward heparin-coated perfusion equipment

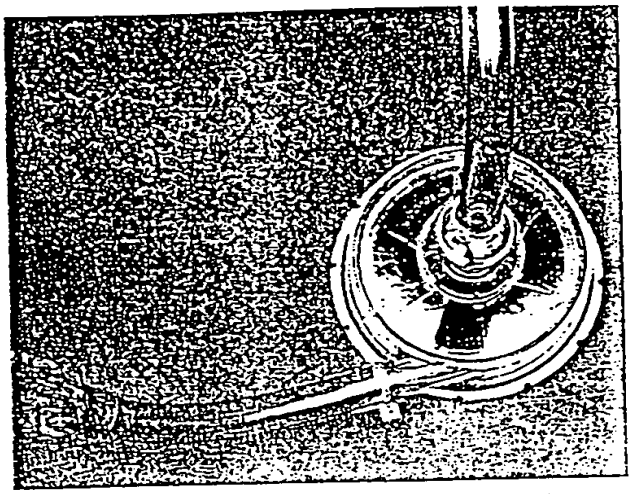

Fig 7. Carmeda Bio-Active System heparin-coated Bio-Medicus pump head carefully rinsed after clinical left heart bypass with low systemic heparinization: clean device.

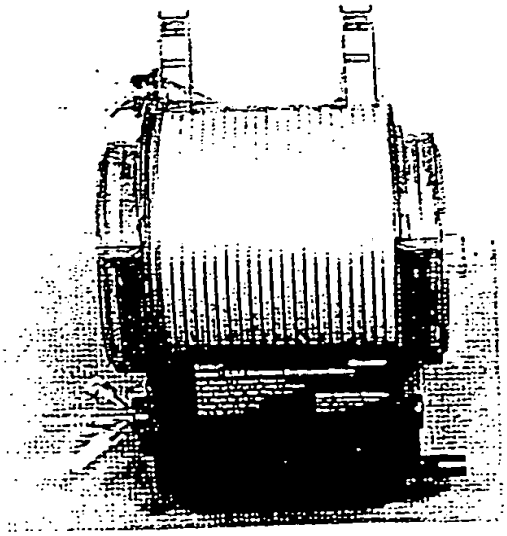

Fig 6. Duraflo II heparin-coated Univox oxygenator carefully rinsed after clinical perfusion with low systemic heparinization: clean device.

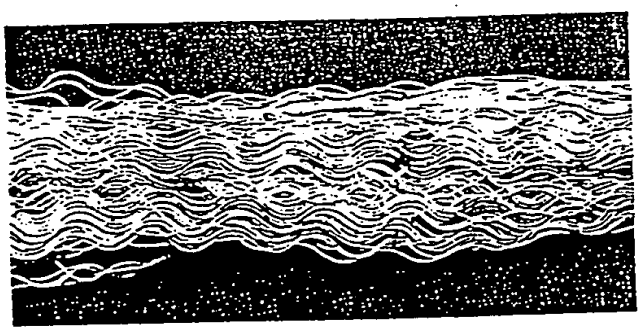

Fig 8. Heparin-coated siliconized microporous hollow fibers after efficient clinical intravascular gas exchange over 29 days: functional device.

weaning from cardiopulmonary bypass after up to 36 hours. Interestingly, the heparin-coated oxygenators functioned well throughout the perfusion period without systemic heparinization. Furthermore, hemodynamics of the animals perfused without systemic heparinization were far more stable than hemodynamics of those perfused with full systemic heparinization. Significantly higher blood loss was observed for the group perfused with full systemic heparinization. Hence, in the absence of homologous transfusions, perfusion using heparin-coated equipment without systemic heparinization allowed us to postpone the usual complications linked to venoarterial cardiopulmonary bypass with full systemic heparinization.

Closed Chest, Partial Cardiopulmonary Bypass With Heparin-Coated Equipment

Toomasian and colleagues [8] evaluated Duraflo II-coated ECMO circuits in sheep. Venovenous extracorporeal circulation was maintained for 4 days. No heparin was given before or during 96 hours of perfusion. Heparin, 20,000 U, was given after the 96-hour perfusion period only before the animals were electively sacrificed. There was no bleeding, no major thrombosis in the circuits, and no significant emboli after 4 days of extracorporeal circulation without anticoagulation.

Venovenous bypass using Carmeda Bio-Active System (CBAS; Carmeda, Stockholm, Sweden) heparin-coated oxygenators (Maxima; Medtronic, Anaheim, CA) without systemic heparinization in conscious sheep up to 5 days was reported by Motthagy and associates [9]. At no time was heparin administered. No complications occurred. Partial thromboplastin time and activated coagulation time remained within the physiologic range. Platelet count did not drop to less than 80% of baseline values and hemolysis rates were negligible, whereas blood gas data remained normal during the entire extracorporeal circulation period. Interestingly, no plasma leakage resulting in decreased oxygenator performance was observed.

Similar results were reported by Marcolin and colleagues [10] for CBAS-coated oxygenators with an extracorporeal blood flow of 500 mL/min and recirculation of 2,000 mL/min provided by a second pump to maintain high oxygenator blood flows. The selected heparinized surface allowed them to maintain 24-hour bypass with normal coagulation times at low circulating heparin levels.

Figure 1 shows a CBAS-coated Maxima oxygenator with transparent housing that was rinsed after arteriovenous perfusion in bovine experiments. There are only minimal macroscopic deposits in the heat exchanger section.

Roller Pump Perfusion With Heparin-Coated Pump Loop

Roller pump left heart bypass based on multiple Gott shunt assembly was our initial approach to active shunt-multiple surface coatings including tridodecylmethylammonium chloride, Duraflo II [11], CBAS [2], and finally phospholipids mimicking the membranes of blood cells [12] were studied. Improved thromboresistance was documented for all the surface modifications cited.

Saito and colleagues [13] reported on 24-hour canine experiments using a seamless construction with heparinized hydrophilic polymer (Anthron; Toray Industries, Tokyo, Japan) and a pump flow of approximately 30% of cardiac output. No thrombus formation was observed in the tubing sets after 24 hours of bypass, and there were no significant changes in activated coagulation time, prothrombin time, fibrinogen level, and plasma hemoglobin level. The level of fibrinogen degradation products remained less than 10 μg/mL. No emboli were found at autopsy.

Centrifugal Pump Perfusion With Heparin-Coated Pump Heads

Left heart bypass using CBAS heparin-coated centrifugal pump heads (Bio-Medicus; Medtronic, Eden Prairie, MN) and heparin-coated tubing sets with flows up to 4 L/min was realized in our laboratories in bovine studies [14]. Reduced blood loss despite open chests, improved fluid balance, and superior mixed venous oxygen saturation were found for heparin-coated equipment without systemic heparinization in comparison with standard equipment with full systemic heparinization.

Van der Hulst and colleagues [15] looked at long-term centrifugal right heart bypass using Duraflo II heparin-coated centrifugal pump heads versus uncoated controls without systemic heparinization. Low flows between 400 and 850 mL/min over 24 hours were used. Both coated and uncoated circuits remained patent at low flows. However, pulmonary emboli were found in some animals of both groups (coated as well as uncoated pump heads).

We are studying the spinning impeller Sarns centrifugal pump (Sarns 3M, Ann Arbor, MI) with Duraflo II heparin surface coating versus uncoated controls in bovine experiments without systemic heparinization and flows up to 4 L/min (unpublished results). These pump heads allow for a 50% reduction in priming volume in comparison with the above-mentioned Bio-Medicus pump heads and show promising preliminary results. A Duraflo II coated pump head and an uncoated control, rinsed after perfusion without systemic heparinization, are shown in Figures 2 and 3. After the challenging bovine experiments without systemic heparinization there is a clear difference with regard to impeller deposits in favor of the heparin-coated pump head.

Matsuwaka and colleagues [16] reported an experimental study evaluating heparin-coated pediatric centrifugal pumps in combination with a hollow-fiber membrane oxygenator. The simplified circuit allowed for perfusion without a venous reservoir for 12 hours with a bypass flow of 500 mL/min. Efficient gas exchange and

with improved thromboresistance to be able to reduce the need for systemic heparinization.

Our clinical experience with heparin-coated devices includes more than 300 patients who underwent mainly total cardiopulmonary bypass for open heart operations [21] or partial cardiopulmonary bypass for proximal unloading and distal protection during repair of descending thoracic aortic aneurysms [22]. Duraflo II heparin-coated systems (Fig 6) have been preferred because of their little extra cost. In both cited studies, reduction of systemic heparinization was closely linked to reduced postoperative blood loss, which in turn allowed for fewer transfusions and fewer surgical revisions for hemostasis. Centrifugal pumps with heparin-coated pump heads (Fig 7) were used for various types of support in 30 patients up to 6 days.

However, the longest experiences with heparin-coated devices exposed to the blood stream we are aware of were achieved with intravascular gas exchangers. One single intravascular gas exchanger with heparin surface coating implanted in the caval axis remained exposed to the blood stream for 29 days [23]. A photographic view of the explanted and carefully rinsed device is shown in Figure 8. This experience demonstrates that longer applications of heparin-coated surfaces are feasible. Interestingly, the membrane used in the intravascular gas exchanger [24] is based on a siliconized microporous hollow fiber that is heparin coated. Significant gas exchange without plasma leakage was obvious throughout the 29-day implantation period for this clinical case, who is a long-term survivor.

Walter Dembitsky (Sharp Memorial Hospital, San Diego, CA; personal communication) has had 11 patients who underwent ECMO for cardiac support with heparin-coated equipment. In his series, the longest perfusion was 119 hours 40 minutes and 30-day survival was 4 of 11 (36%).

Lazzara and colleagues [25] reported on ECMO for postcardiotomy cardiogenic shock in 11 adults using heparin-coated systems (CBAS heparin coating, Maxima oxygenator, Bio-Medicus vortex pump). Mean duration of support was 47.9 hours (range, 22.0 to 92.5 hours). In this group circulating heparin was neutralized with protamine. No additional heparin was given for pump flows greater than 1 L/min. During perfusion, mean prothrombin time was 17 ± 8 seconds, mean activated partial thromboplastin time was 57.5 ± 38 seconds, and activated coagulation time was 152 ± 59 seconds. Despite perfusion without additional heparinization, bleeding was the most frequent complication followed by hemolysis and mechanical oxygenator change. Of the 11 patients, 8 (73%) were weaned and 6 (55%) were discharged. Five (45%) are long-term survivors. Unfortunately, the information given by Lazzara and colleagues does not allow us to identify the cause of the described gradual oxygenator failures requiring device replacement during ECMO. We know from ECMO in patients with adult respiratory distress syndrome that progressive plasma leakage with decreasing oxygenator performance is a common problem of microporous hollow-fiber membrane oxygenators even with heparin coating. Lazzara and colleagues state, however, that the heparin-coated oxygenators were not inert and activated the inflammatory or clotting cascade. The latter could be due to the fact that protamine was used during perfusion with heparin-coated equipment. Similar problems, ie, clots on heparin-coated surfaces, were reported by Bianchi and associates [26], who had also used protamine during perfusion with heparin-coated centrifugal pumps. We have previously shown in ex-vivo experiments that protamine given during perfusion with heparin-coated equipment reduces the antithrombotic activity of bonded heparin and should therefore be avoided [27]. Use of fresh frozen plasma, coagulation factors, platelet concentrates, and aprotinin can help to avoid protamine use during perfusion with heparin-coated equipment. Alternatively, an extracorporeal heparin removal device used with a heparin-coated tubing set allows for absorption of circulating heparin on immobilized polycation and reactivates the coagulation system [28].

In conclusion, heparin surface coatings have been shown to improve the thromboresistance of blood-exposed surfaces of circulatory support devices and to allow for reduction of systemic anticoagulation during perfusion with consecutive reduction of bleeding complications. Furthermore, heparin surface coating can also be applied to leak-proof gas exchangers with true membranes. However, thromboresistance of heparin surface coatings is dependent on blood flow and can be reduced by contamination with protamine.

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Heparin-coating of bypass circuits

Principles of heparin-coating techniques

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Introduction

Exposure of blood to a foreign surface results in plasma protein alterations and blood cell activation. Extracorporeal blood circulation is only possible with the use of systemic anticoagulants, such as heparin, to inhibit the clotting system. Even with the use of high doses of heparin, clinical cardiopulmonary bypass (CPB) and other extracorporeal procedures have been associated with intra- and postoperative bleeding,[1] pulmonary abnormalities,[2] neurological dysfunction[3] and organ damage. Such complications have been attributed to impaired haemostatic functions, thromboemboli formation,[4] leukocyte and platelet activation,[5] and complement activation, all of which may be related to the blood material interactions.

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Despite the intensive research in the area of blood compatible materials or surfaces in the past decades, the most blood compatible surface remains to be the endothelial lining. Recent evidence[6] suggests the presence of heparin-like molecules (heparin sulphate) along with other biologically active substances on the luminal surface of the microvasculature endothelium. The endothelium may actively interact with blood when the coagulation status of the circulating blood changes. Surfaces incorporating biologically active substances, thus, appear to be a more viable approach to blood compatible surfaces. Among the various biologically active substances that have been immobilized onto synthetic surfaces, heparin-coating or immobilization is the most well explored and promising approach.

Heparin is a natural sulphated glycosaminoglycan consisting largely of alternating L-iduronic acid and N-sulphated D-glucosamine residues. Heparin exerts its anticoagulant activities through interaction with antithrombin III (AT III). When heparin binds to AT III, the complex causes an

acceleration of the action of AT III in inactivating thrombin (IIa), factor IXa, factor Xa, factor XIa, factor XIIa and plasma kallikrein.[7] Recent studies suggest that only small portions of the heparin molecule that contain specific carbohydrate sequences are responsible for the anticoagulant activities. A pentasaccharide sequence, through its action with AT III, is responsible for inhibiting factor Xa. A sequence containing 18 saccharide units is believed necessary for thrombin inhibition.[8] When heparin is modified for surface coating or immobilization, it is essential that the active sequences responsible for anticoagulation are not altered during the modification.

Techniques of heparin-coating

Numerous techniques have been utilized to immobilize heparin onto synthetic surfaces. The techniques generally fall into the following categories: ionically bonded heparin, covalently bonded heparin and physically entrapped heparin.

Ionically bonded heparin

Since heparin contains a large number of negatively charged groups, synthetic surfaces containing positive charges will attract heparin molecules onto the surfaces through electrostatic attraction. Positive charges can be introduced onto the surfaces by the use of a cationic surfactant or polymer coating containing cationic groups.

The first surface bonded to heparin was reported by Gott[9] in 1963. The work involved the attachment of heparin through formation of a complex with a quaternary ammonium salt, benzalkonium chloride, that was adsorbed onto graphite. Graphite benzalkonium heparin-coated vena cava rings implanted in dogs remained thrombus-free for more than two weeks. At present benzalkonium heparin (without graphite) is still the most widely used heparin-coating material for clinical devices. Benzalkonium heparin, while having excellent thromboresistant properties, has limited applications for extracorporeal devices that require priming with saline before blood contact. It is fairly soluble in saline and leaches off the surface during prime. A complex of tridodecylmethylammonium chloride and heparin (TDMAC heparin) was developed by Grode[10] to improve the applicability of coating to a variety of different synthetic surfaces. TDMAC-heparin-coated shunts have been used clinically for temporary bypass of thoracic aneurysms.

Cationic groups, such as N,N-dimethylamino-ethyl groups, have been incorporated into a polymer matrix to facilitate heparin adsorption.[11] Polyamido-amine chains, capable of complexing with heparin molecules, have been attached to polyurethane and glass surfaces.[12]

Covalently bonded heparin

Heparin has been linked directly to synthetic surfaces to achieve stable bonds. Carbodiimides have been used to link heparin to hydrolyzed polymethylmethacrylate.[13] Heparin has been covalently bonded to polyvinylalcohol by using glutaraldehyde.[14] Covalent attachment of heparin requires specific chemistry to be developed between the heparin and a surface. For an extracorporeal device, a separate process for each surface/material in the device would be required. Covalent heparin bonding is thus often time consuming and, in some cases, could result in physical damage of the surfaces of the device. The covalently bonded heparin has been widely used as an adsorbent in affinity chromatography. A reduction of heparin activity is frequently observed when heparin is covalently attached to the surface. Presumably, the active carbohydrate sequences are altered in the covalent linking process.

Several polymer coatings containing covalently attached heparin have been reported. Heparin-polyvinylalcohol hydrogel coating,[14] poly dimethylsiloxane-polyethylene oxide-heparin block copolymer[15] and albumin-heparin conjugates[16] have been prepared and characterized.

Physically entrapped heparin

Heparin molecules have been incorporated into a polymer binder to provide slow release of heparin upon blood contact. A physical blend of heparin and polyurethane[17] has been evaluated as a coating material for catheter applications.

Heparin-coating for cardiopulmonary devices

Heparin-coating for cardiopulmonary devices

presents specific challenges. Devices such as oxygenators, cardiotomy reservoirs, blood filters, etc. are of complex configurations and have substantial surface areas. They are made from different synthetic materials including polycarbonate (housing), polypropylene (hollow fibre membrane), plasticized polyvinyl chloride (tubing and cannulae), polyester (filter), polyurethane (defoamer), stainless steel (heat exchanger) and many others. Coating stability, toxicity, thromboresistance and compatibility with different surfaces must be taken into consideration.

Two processes have recently been applied to cardiopulmonary bypass circuits. One process is based on the concept of 'universal surface'. This is accomplished by depositing or ionically attaching a polymer coating, polyethyleneimine (PEI), onto various types of surfaces. Heparin fragments, prepared from the degradation of heparin in nitrous acid, were then attached to the PEI.[18] The other process is based on the concept of 'universal coating', whereby the physicochemical properties of unfractionated heparin are modified with a proprietary binding agent. The resulting material, trade-named Duraflo II heparin, has high affinity to a variety of synthetic surfaces and retains all anticoagulant properties of heparin.

Evaluation of heparin-coated surfaces

Earlier attempts to evaluate the thromboresistance of heparin-coated surfaces included animal implantation of heparin-coated devices and setting up heparin-coated extracorporeal circuits.[19] Due to the complexity of heparin immobilization techniques, the consistency of coating processes, surface characteristics of different substrates and potential toxicity associated with the coating materials, the results have been inconsistent. Until very recently, clinical uses of heparin-coated devices have been limited to relatively simple devices such as pulmonary artery catheters and shunts.

Arterial filters

Heparin-coated arterial filters have recently gained increasing popularity in clinical use. The perceived advantages, however, are the ease of priming and debubbling due to the hydrophilic nature of heparin-coated surfaces. To evaluate the effect of various heparin coatings on arterial filters, an *in vitro* blood recirculation loop test shown in Figure 1 was developed. A monolayer of filter secreen (8.3cm^2) was sealed in a filter housing and the circuit was primed and recirculated with saline. Freshly drawn bovine blood, anticoagulated with 4 units heparin/mL

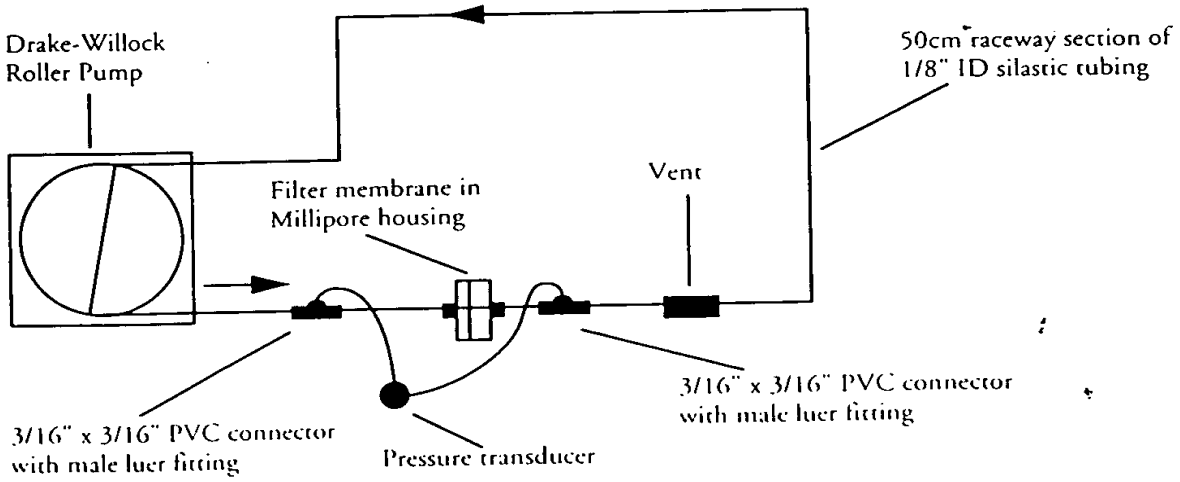


Figure 1 *In vitro* blood recirculation loop test

blood, was introduced and recirculated in the test loop. Platelet depletion, pressure differential across the screen and thrombus formation on the screen were used to assess the blood compatibility of the screens. The filter screens evaluated included the following:

1) 40 microns woven polyester screen.
2) 40 microns woven polyester screens coated with benzalkonium heparin.
3) 40 micron woven polyester screen coated with TDMAC-heparin.
4) 40 micron woven polyester screen coated with Duraflo II heparin.

Typical pressure traces for each of the screens tested are shown in Figure 2. Uncoated polyester screen, benzalkonium heparin-coated screen and TDMAC-heparin-coated screen generally exhibited episodes of inlet pressure increases followed by sudden inlet pressure decreases. No pressure increases or variations were noted on Duraflo II heparin-coated screens. Average % platelet depletion after 30 minutes of blood recirculation is summarized in Table I. Corresponding scanning electron micrographs shown in Figure 3 correlate well with the platelet depletion and inlet pressure data and suggest that the observed pressure increases are related to the deposits of blood components resulting from blood-material interactions. Pressure increase followed by a reduction of pressure has previously been related to the release of thrombi from the arterial filter surfaces.[20] Even though the *in vitro* testing conditions are more vigorous than typical clinical conditions, the results agree with those reported previously by Longmore,[21] Heimbecker[22] and others,[23] who observed increases in inlet pressure, reduction in platelet counts and

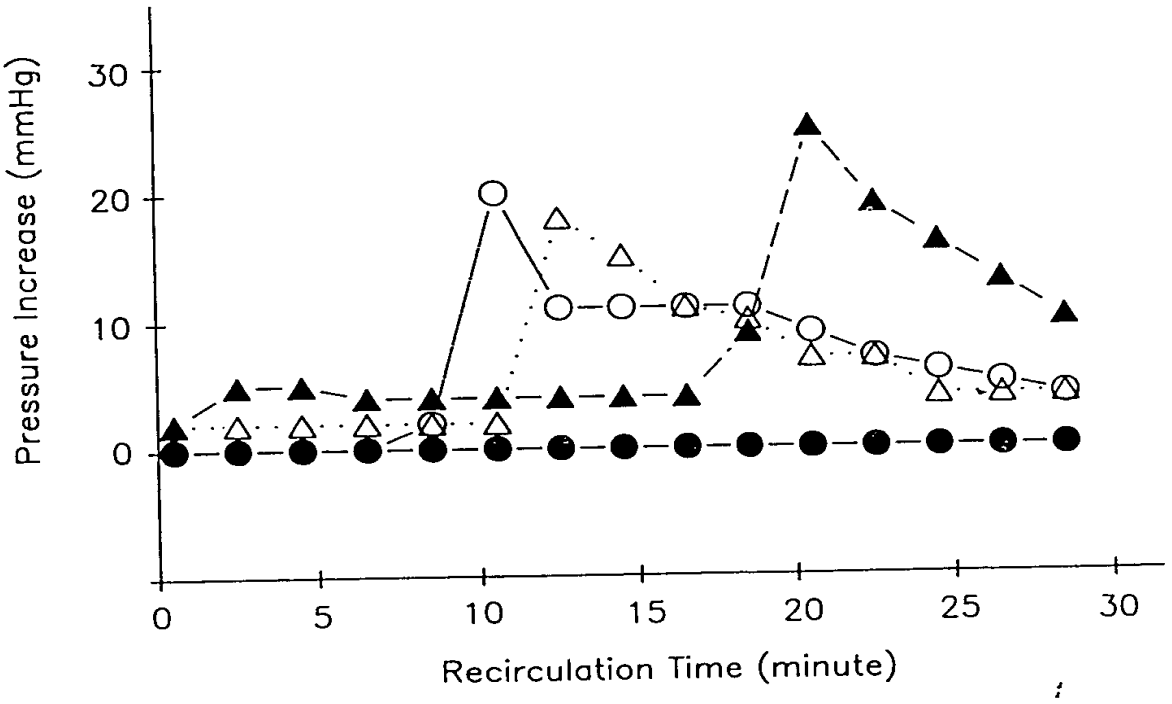
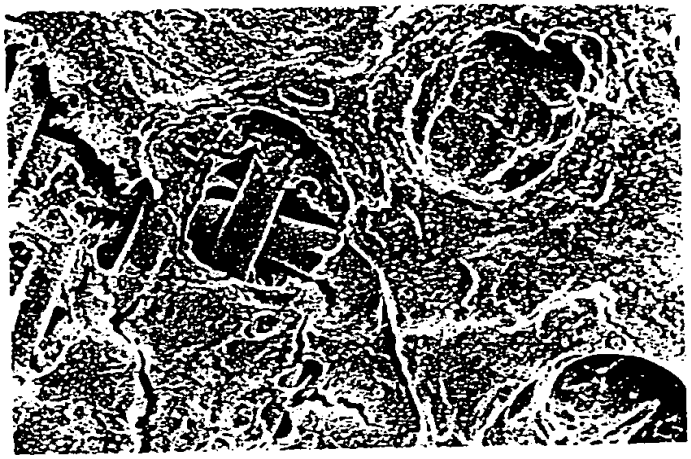


Figure 2 Pressure drop versus blood recirculation time. Bovine blood containing 4 units heparin/ml blood; blood flow rate 100 ml/min.

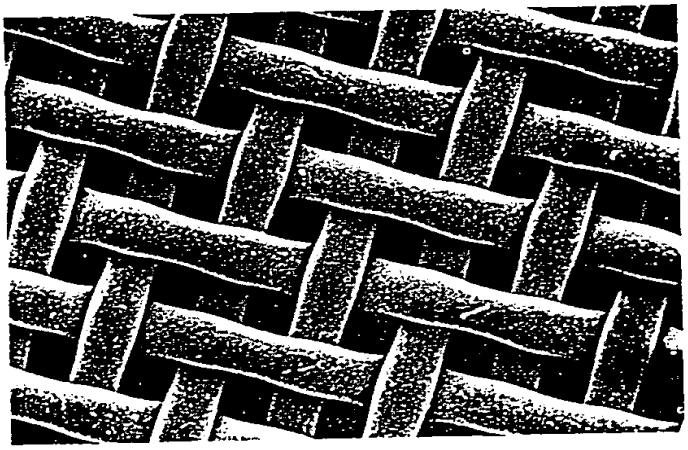
polyester screen = O —— O
polyester screen coated with benzalkonium heparin = △ ··· △
polyester screen coated with TDMAC-heparin = ▲ —— ▲
polyester screen coated with Duraflo II heparin = ● —— ●

3a: Polyester screen



3b: Polyester screen coated with benzalkonium heparin

3c: Polyester screen coated with TDMAC-heparin



3d: Polyester screen coated with Duraflo II heparin

Figure 3 Scanning electron micrographs of screens after 30 minutes of blood recirculation

Table 1 Average platelet depletion data from *in vitro* blood recirculation loop test

	PES (n=6)	BKH (n=6)	TDMAH (n=3)	DURAFLO II (n=6)
Baseline platelet count ($\times 10^3/\mu L$)	523±51	459±23	514±50	528±33
Final platelet count after recirculation ($\times 10^3/\mu L$)	46±25	74±120	113±52	408±40
% of platelet depletion from baseline	91±5%	62±26%	78±11%	23±6%

Bovine blood containing 4 units heparin-ml blood recirculated at flow rate of 100 ml/min for 30 minutes.

PES = polyester; BKH = benzalkonium heparin-coated polyester; TDMAH = TDMAC-heparin-coated polyester; DURAFLO II = Duraflo II heparin-coated polyester.

deposition of platelets during clinical and experimental uses of the filters. The results from the *in vitro* blood recirculation, thus, strongly suggest that the principal benefits of properly heparin-coated arterial filters are improved blood compatibility, reduced blood-material interactions and prevention of microemboli.

The ineffectiveness of benzalkonium heparin-coated screen is a result of heparin leaching during the priming and recirculating with saline before blood contact is made. The stability of benzalkonium heparin on arterial filters was compared with that of Duraflo II heparin-coated arterial filters by recirculating saline through the filters at 6 l/min for two hours. The amount of heparin retained on the filters and the amount leached into circulating saline were monitored using a spectrophotometric technique and Factor Xa inhibition method. Normalized leaching rates for the filters analysed are shown in Figure 4. Nearly all benzalkonium heparin leached off the surface during 15 minutes of saline recirculation. The heparin leaching from commercially available arterial filters was recently determined by monitoring the surface tension of priming solution.[24]

Other materials

Platelet adhesion properties of polypropylene (PP) and heparin-coated polypropylene surfaces were evaluted *in vitro* using a Chandler loop with heparinized bovine blood containing Cr-51 labelled platlets.[25] Significant reductions in both platelet adhesion and platelet depletion were noted with the heparin-coated PP surface (Table 2). An *ex vivo* canine arteriovenous series shunt technique[26] was used to evaluate the platelet compatibility of the heparin-coated surface.

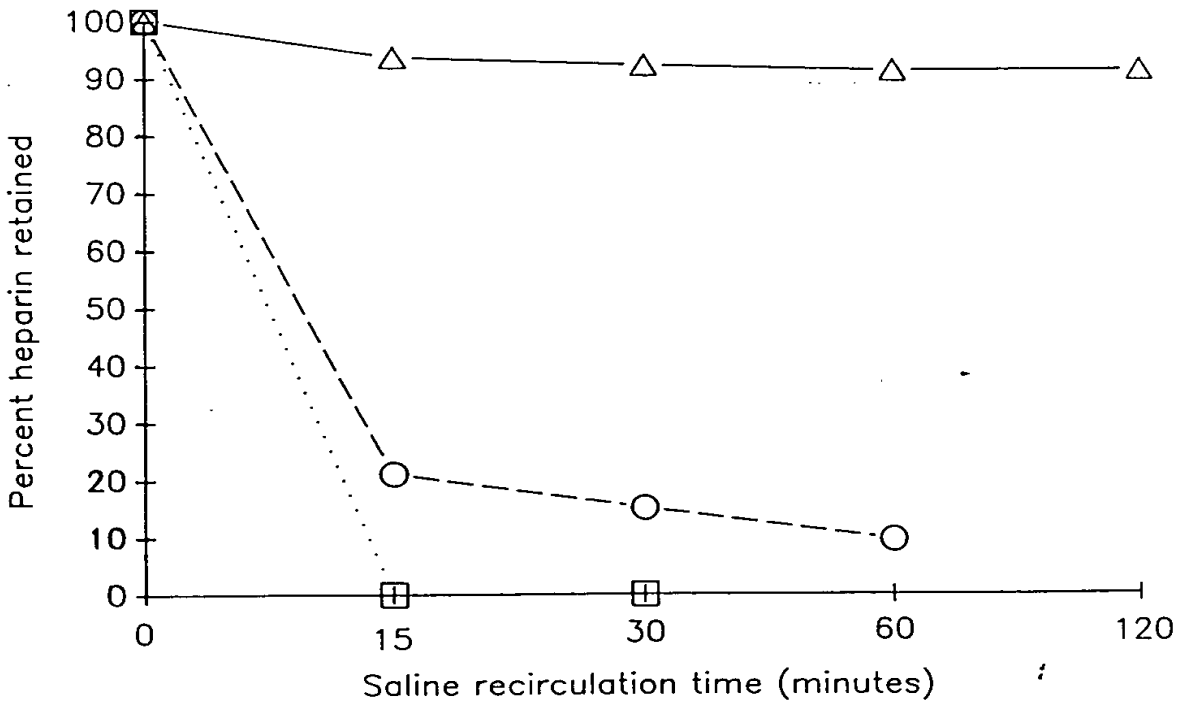


Figure 4 Stability of heparin on arterial filters

benzalkonium heparin-coated filter (commercial filter A) = O—— O
benzalkonium heparin-coated filter (commercial filter B) = □ ··· □
Duraflo II heparin-coated arterial filter = △ —— △

Figure 5 shows qualitatively the amount and integrity of platelets adhered onto a standard PVC and heparin-coated PVC tubing material after 20 minutes of blood recirculation with a nonheparinized canine.

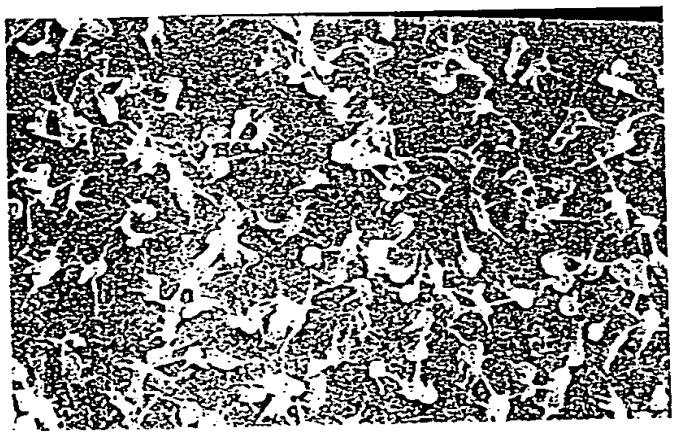
Oxygenators

The thromboresistance of heparin-coated oxygenators was compared with that of uncoated oxygenators *ex vivo* using a bifurcated circuit. Venous blood from a jugular vein of a test animal was drained and bifurcated into two oxygenators connected in parallel, one coated with Duraflo II heparin and the other was an uncoated oxygenator. The oxygenated blood was then combined and returned to the animal. The circuit essentially eliminates the animal to animal variation and allows a direct comparison to be made between coated and uncoated oxygenators. Thrombus formation was compared on microporous hollow-fibre membrane oxygenators (Figure 6) after six hours of venoarterial partial cardiopulmonary bypass on calves with activated clotting times (ACT) maintained at 240 seconds. The effectiveness of heparin coating was also demonstrated in a similar study[27] comparing a Duraflo II heparin-coated silicone membrane oxygenator with an uncoated oxygenator after six hours venoarterial bypass on calves in which systemic anticoagulant was eliminated (Figure 7). In both cases little or no clots were observed in the coated oxygenators as compared to extensive thrombus formation in the control uncoated oxygenators. It is interesting to note that the amounts of Duraflo II heparin immobilized on the coated microporous oxygenator and coated

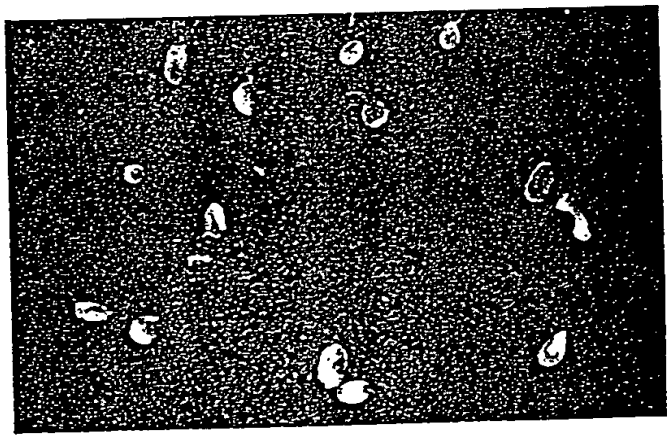
Table 2 Cr-51 labelled platelet adhesion and platelet depletion

Time (min)	Platelet adhesion ($\times 10^5$/cm^2, n=3) PP	PP/D-II	% Platelet depletion from baseline (n=3) PP	PP/D-II
5	8.0±0.4	1.0±1.0	16.4±4.2%	7.8±4.8%
20	24.0±2.4	2.1±0.1	33.7±7.5%	10.6±4.4%
60	46.0±4.9	6.3±2.1	65.5±6.8%	20.0±3.5%

Heparinized bovine blood (4 units heparin/ml blood) containing labelled platelets recirculated in a Chandler loop consisting of polypropylene (PP) tubing or Duraflo II heparin-coated PP (PP/D-II) tubing.

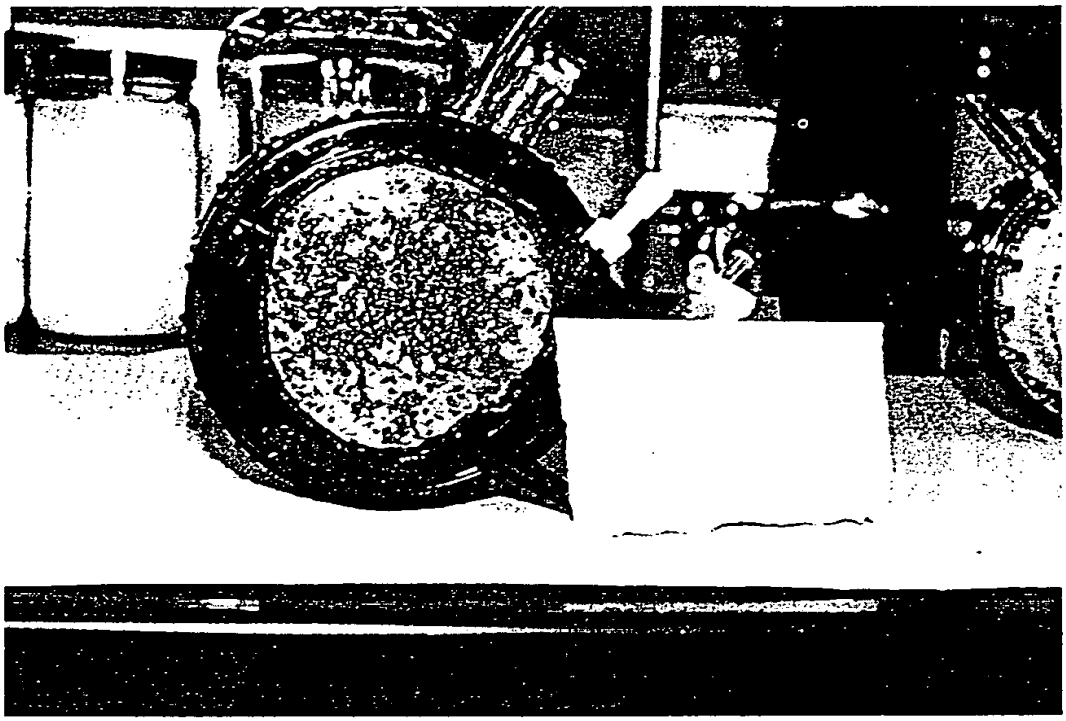


5a: Standard PVC tubing

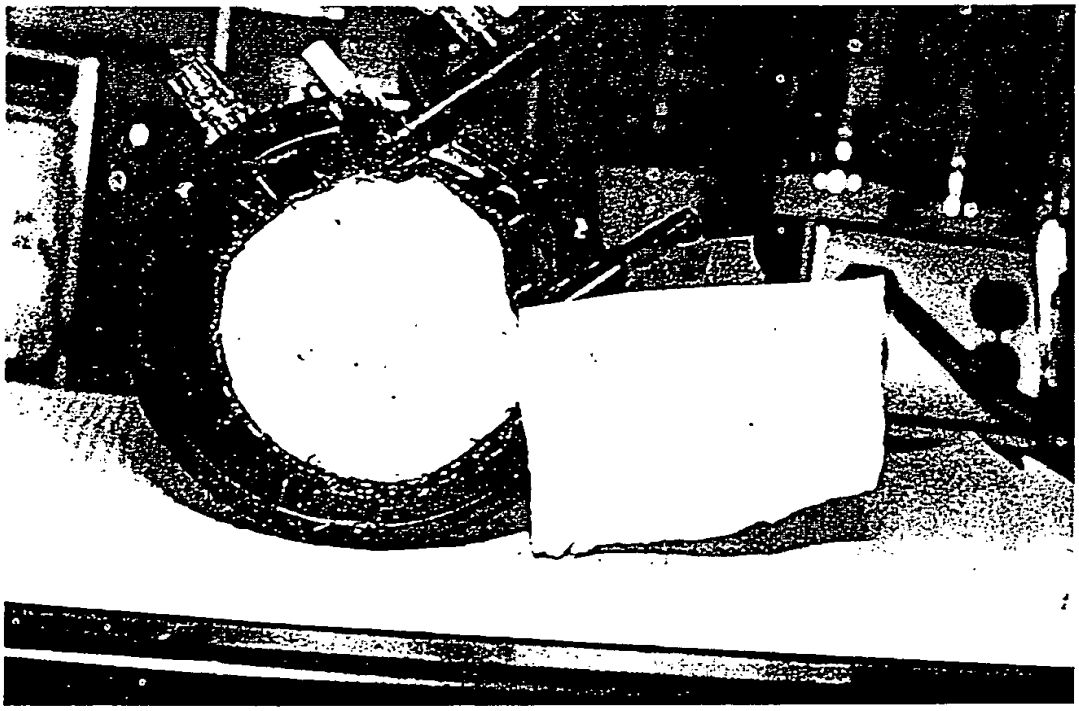


5b: Duraflo II heparin-coated PVC tubing

Figure 5 Scanning electron micrographs showing platelet adherents after 20 minutes of blood circulation — no systemic heparin used.



6a: Uncoated oxygenator



6b: Duraflo II heparin-coated oxygenator

Figure 6 Outlets of microporous hollow-fibre oxygenators from a bifurcated circuit after six hours VA partial bypass on bovine. ACT 240 sec, blood flow rate 3.5 l/min each.

silicone membrane oxygenator shown in Figures 6 and 7 were approximately 1000 USP units and 600 USP units, respectively. Control experiments in which the test animals were systemically anticoagulated with similar amounts of heparin with uncoated circuits resulted in severe clotting in the oxygenators. The lack of thrombus formation in the heparin-coated oxygenators suggest that immobilized heparin is many times more effective in inhibiting the clotting system than the circulating heparin.

Cardiopulmonary bypass circuits

The effect of fully heparin-coated cardiopulmonary bypass circuits has also been determined in calves under simulated clinical conditions. Calves were placed on CPB for four hours and the ACTs were maintained above 480 seconds with systemic heparin. Better preserved platelet number and function, lower fibrinopeptide A generation and lack of thrombus deposition were observed in the heparin-coated group relative to the uncoated control group.[28] In a canine study of lung dysfunction following open heart surgery, Taylor[29] demonstrated that the white cell activation and sequestration of white cells within the lung was substantially reduced with the use of the heparin-coated circuits.

Heparinless bypass

The possibility of eliminating systemic anticoagulant with the use of heparin-coated circuits for cardiopulmonary bypass and prolonged extracorporeal membrane oxygenation has been investigated. von Segesser *et al.*[30] successfully performed 24 hours heparinless cardiopulmonary bypass on canines using Duraflo II heparin-coated circuits. Toomasian *et al.*[31] reported that, with the use of fully Duraflo II heparin-coated circuits, it was possible to perform heparinless ECMO on sheep for four days without complications.

While the immobilized heparin appears to be many times more effective than the systemic heparin, the mechanism by which the immobilized heparin operates is not fully understood. It is possible that heparin placed on the sites of blood activation (foreign surfaces)

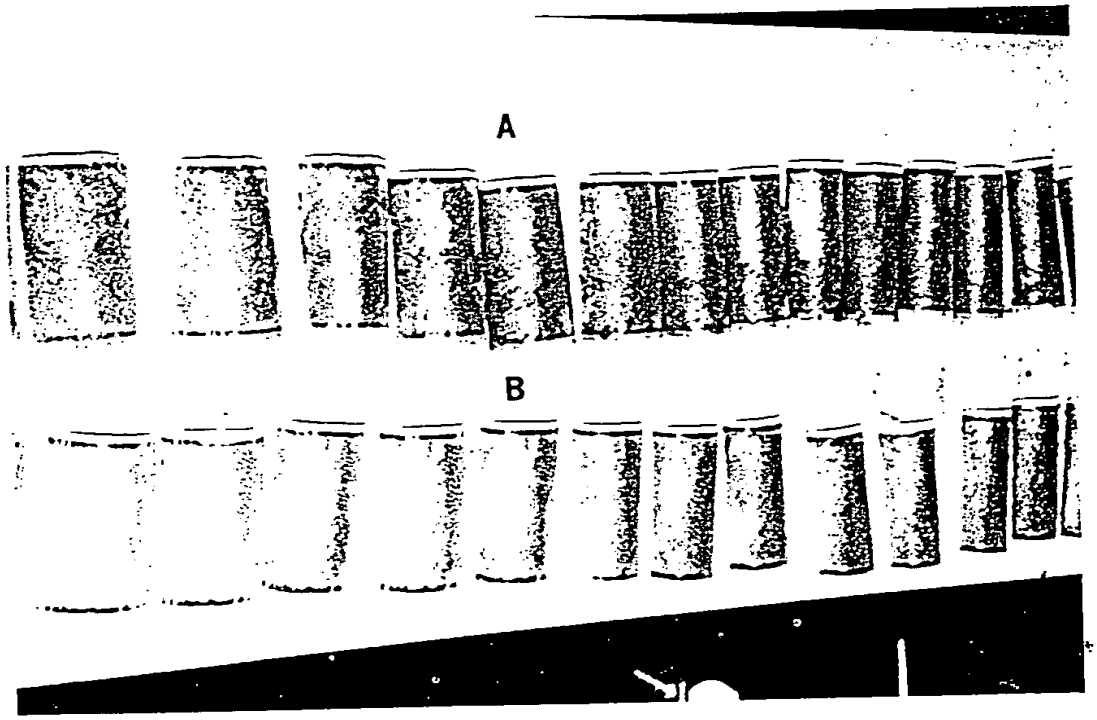


Figure 7 Silicone membrane oxygenators from a bifurcated circuit after six hours VA partial bypass on bovine No systemic heparin used; blood flow rate 1 l/min each: A = uncoated oxygenator; B = Duraflo II heparin-coated oxygenator.

may reduce contact activation as heparin/AT III, in addition to inhibiting factors IIa and Xa, also inhibits factor XIIa, plasma kallikrein and factors XIa and IXa. Heparin coating may also alter the normal sequences of plasma protein deposition on surface. The protein 'passivated' surfaces may be ultimately responsible for the long-term blood compatibility. The clinical significance of heparin-coated extracorporeal circuits is being explored at this time. To fully utilize the potential benefits of blood compatible surfaces, it is important that the devices must have appropriate haemodynamic designs to avoid blood stagnation and high shear stress.

Conclusions

Blood compatibility has long been regarded as a critical requirement for blood contact devices. *In vitro* and *ex vivo* results suggest that heparin-coated devices offer significantly improved blood compatibility. The improvement may reduce the clinical complications currently associated with CPB and other extracorporeal bypass procedures and enhance safety of bypass procedures. Improved thromboresistance may allow modification of systemic anticoagulation levels during bypass to further reduce complications associated with the use of high doses of anticoagulants.[32]

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Evaluation of Duraflo II Heparin Coating in Prolonged Extracorporeal Membrane Oxygenation

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Adult sized extracorporeal membrane oxygenation circuits were coated with Duraflo II chemical heparin bonding by Baxter-Bentley Laboratories. Five sheep were maintained on venovenous extracorporeal circulation for four days with no systemic anticoagulation. There was no bleeding, no major thrombosis in the circuits, and no significant emboli after 4 days of extracorporeal circulation without anticoagulation. ***ASAIO Transactions*** **1988; 34: 410–414.**

Routine clinical use of extracorporeal circulation for renal, cardiovascular, and respiratory diseases, as well as for some organ transplants, requires systemic heparin in order to prevent extracorporeal circuit thrombosis. Each procedure has potential side effects which include bleeding, coagulation factor consumption, and thrombocytopenia. Although alternatives to anticoagulation such as platelet inhibiting drugs and heparin coated nonthrombogenic circuits have been studied, no alternative to systemic heparinization has been developed and used in routine clinical procedures. A variety of immobilized heparin-coated systems have been evaluated, but are limited in their use due to cost, chemical complexity, biomaterial binding irregularity, and irregular flow patterns of the extracorporeal circuit. The current study reports on hematologic and activated clotting time changes with a new heparin-bonded surface in five sheep supported on venovenous extracorporeal membrane oxygenation (ECMO) for 96 hours.

Methods

Five sheep weighing 33 to 40 kg were anesthetized with ketamine (10 mg/kg), intubated, and ventilated with room air (14 ml/kg). A stomach tube was inserted to decompress the bowel. Anesthesia was maintained with supplemental doses of ketamine. The right femoral artery was cannulated and used as a source of arterial blood samples and systemic blood pressure. The animals were cannulated for venovenous bypass through the right internal jugular vein and the right femoral vein with polyvinylchloride (PVC) catheters coated with Duraflo II. Cannulae were secured and the animal allowed to revive and stand in a cage.

The extracorporeal circuit components were coated with Duraflo II™ heparin coating complex (Baxter-Bentley Laboratories, Irvine, CA). The circuit consisted of either $^3/_8 \times {}^3/_{32}$ inch and/or $^1/_4 \times {}^1/_{16}$ inch Bentley PVC tubing, polycarbonate connectors, a silicone rubber bladder, and a Bentley CM-40 polypropylene hollow fiber membrane lung with integral heat exchanger.

The heparin complex was dissolved in 1,1,2,trichloro 1,2,2,trifluoro-ethane to obtain a coating solution of desirable concentration. The mixture was pumped through the extracorporeal components, drained, and dried. No stress cracking or swelling of the plastics was observed, and the residual solvent was readily and thoroughly evacuated. The total amount of Duraflo II coating on each circuit was equivalent to approximately 2,000 USP heparin units.

The extracorporeal circuit was assembled and flushed with carbon dioxide, primed with 0.9% sodium chloride, and debubbled. The prime was recirculated through the circuit for sixty minutes at a 1 L flow. Prime samples were taken periodically to check for heparin elution by assaying for thrombin inhibition (Protopath Baxter Dade) and factor Xa inhibition. The crystalloid prime was displaced with one unit of fresh citrated sheep whole blood. No heparin was added to the prime. The prime was adjusted for pH with tris(hydroxymethyl) aminomethane (THAM) and recirculated through the A–V bridge to prevent stagnation within the circuit.

Each animal was connected to the circuit, and venovenous perfusion began at flow rates ranging from 15 to 50 ml/kg/min. Pre- and postmembrane lung gases were measured every eight hours. Blood flow rates were maintained at those settings for the duration of the trial. Platelet and leukocyte counts were measured at 30 minutes, 1, 2, 4, 6, 8, 12, 18, 24, and every 12 hours thereafter for 96 hours until the trial was completed. Activated clotting time was measured every hour by the method of Baden *et al.*[1]

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Following 96 hours of extracorporeal circulation, each animal was anticoagulated with 20,000 USP heparin. The animal was killed, decannulated, and autopsied. The heart and lungs were inspected for evidence of thrombosis. The circuit was rinsed with 3 L of .09% sodium chloride, fixed with .02% gluteraldehyde solution, and returned to the manufacturer for additional analysis.

Results

Each animal was alert and awake for the 96 hr bypass, with all animals surviving. Each membrane lung transferred gas adequately; no devices failed, leaked protein, or required replacement during the trial. In each of the membrane lungs tested, the oxygen saturation exceeded 98% and the pCO_2 values were consistently under 35 torr. The membrane lungs were not evaluated for upper flow range gas transfer capability.

Mean activated clotting time for all five animals is shown in **Figure 1.** During the first hour, there was a small increase from baseline (101 ± 17.6 sec–195 ± 60.8 sec). Within 2 hours, the activated coagulation time (ACT) stabilized within 5 to 15% above baseline and was maintained at normal levels (90–180 sec) for the remainder of the trial without supplemental heparin.

The change in mean platelet count expressed as a percent of baseline is shown in **Figure 2.** Platelets were consumed rapidly and were 50% of baseline at 6 hours. Further consumption occurred, although a steady state was reached at a 75% drop from baseline at 48 hours. Visible white platelet aggregates and thrombi were observed on the heat exchanger by 12 to 24 hours of bypass.

The change in leukocyte count expressed as a percent of baseline is shown in **Figure 3.** White blood cells declined initially to under 50% of baseline, but rebounded to nearly twice that of baseline by 8 hours of bypass. Over the next several days, the leukocyte count stabilized and gradually declined toward baseline levels.

Each circuit was inspected after the experiment. The PVC tubing and catheters showed no evidence of thrombosis, the polycarbonate connectors were thrombus free, but some small thrombi were observed in the eddy current areas next to the PVC tubing connections. The silicone rubber bladder showed no visible evidence of thrombosis.

The membrane lung is made of several different biomaterials including: polypropylene, polycarbonate, and polyurethane potting material. The integral heat exchanger is made of convoluted anodized aluminum tubing. Minor to moderate thrombi were found in the oxygenator's stagnant flow areas, in particular the region between the heat ex-

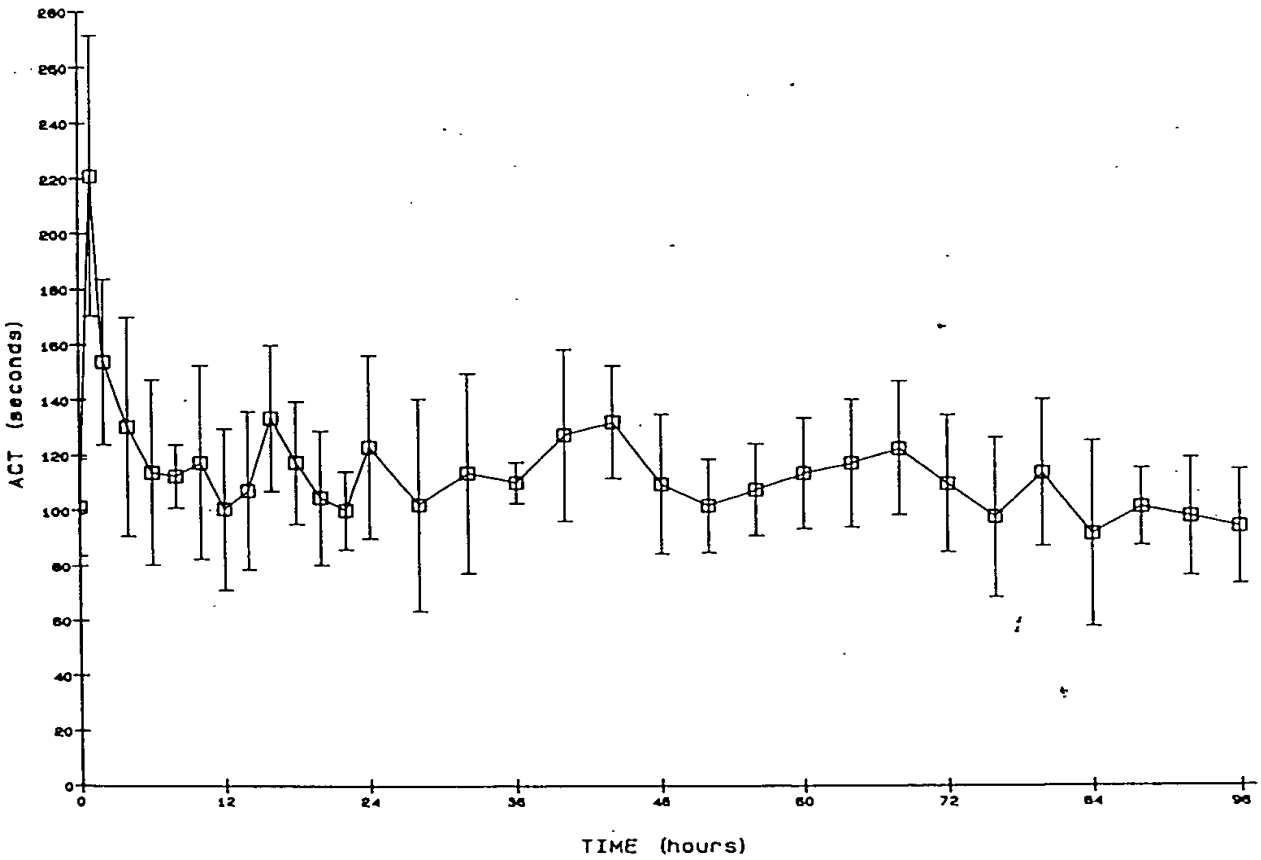

Figure 1. Mean changes (±SD) in activated clotting time in five sheep during 96 hours of venovenous ECMO.

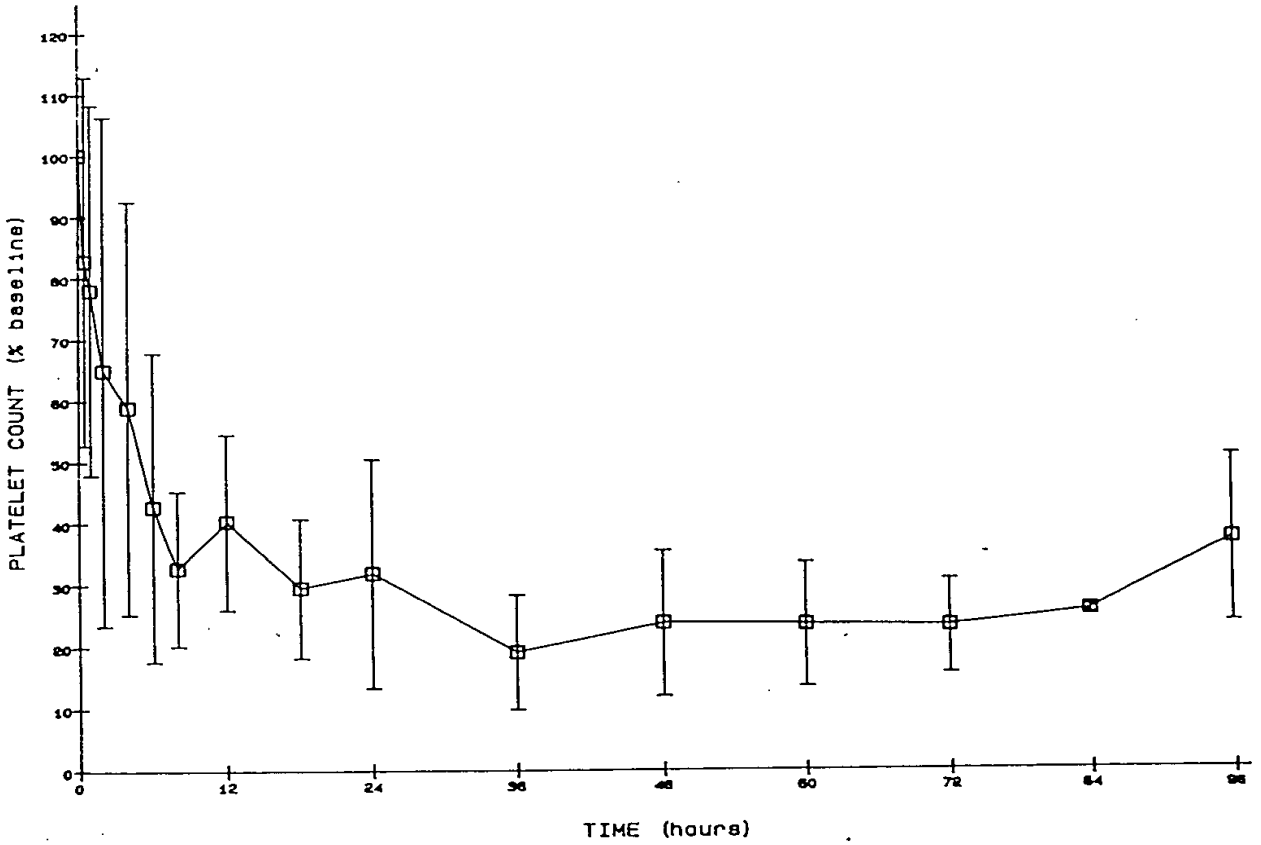

Figure 2. Mean changes (±SD) in platelet count in five sheep during 96 hours of venovenous ECMO, expressed as a percent of baseline.

changer volume displacer and the hollow fibers. Areas of stagnant flow in the heat exchanger were associated with more thrombi, and while the polypropylene hollow fibers were clot free, the outlet header was involved in some stringy thrombi in the periphery of the end cap.

The heart and lungs were examined for gross changes. In all five animals, the atrium and ventricles were thrombus free, and in four of five animals, the lungs showed no evidence of thrombosis. The lungs were pink, and appeared to be well aerated. In one animal, two small thrombi were found in 0.5 cm in diameter pulmonary arteries. In the same animal, there was one small infarcted area of lung (4 × 6 cm) associated with a thrombus.

Discussion

Bleeding, thrombocytopenia, and coagulation factor consumption are major side effects of prolonged extracorporeal circulation with systemic anticoagulation. Development of a nonthrombogenic surface would reduce or eliminate many of the potential risks of bypass and allow for a more widespread use of extracorporeal circulation. Heparin immobilization would be ideal for its natural anticoagulant activity as well as partial endothelial surface characteristics.

Heparin has been ionically and covalently bonded onto several biomaterials. Ionic heparin coatings, such as TDMAC, involve a simple dip coating procedure, and can be applied to a variety of polymeric surfaces through hydrophobic interactions with the substrates. Benzalkonium heparin complexes, however, require different prebonding solvents for different biomaterials. Improper coating can cause stress cracking of harder polycarbonate-type plastics or swelling of silicone rubber. An earlier trial from this group evaluated TDMAC heparin-coated ECMO circuits in sheep for 30 to 48 hours. Activated clotting times were within normal ranges after some initial wash-off of the heparin complex. Thrombosis was observed in areas of stagnant flow and on some larger polycarbonate components.[2] Additional studies have met with some success using benzalkonium heparin-coated surfaces.[3-5]

Covalent heparin bonded surfaces generally require several steps and chemical reactions for each surface to effectively establish the covalent heparin bond. Cottonaro *et al.* described a surface utilizing covalent attachment of a quaternary ammonium compound.[6]

A heparin-coated surface covalently binding heparin to a primary amine that mimics the natural endothelium by taking up fibrin and accelerating plasma antithrombin has been

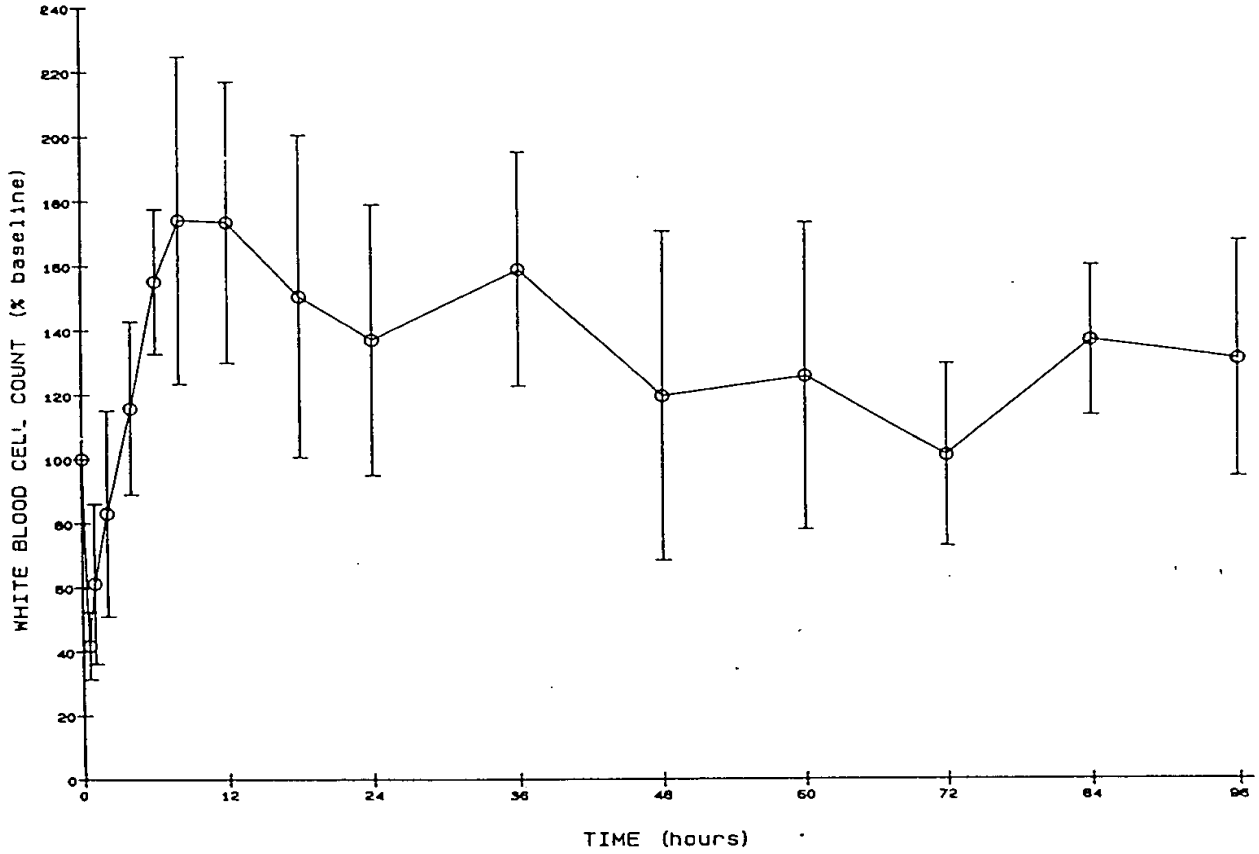

Figure 3. Mean changes (±SD) in leukocytes in five sheep during 96 hours of venovenous ECMO, expressed as a percent of baseline.

reported. Initial trials of this heparin-bonded surface on hemodialysis circuits showed no extracorporeal thrombosis and no dialyzer impairment.[7]

Bindslev and associates[8] evaluated the same covalent heparin coating in six dogs undergoing extracorporeal CO_2 removal for 6 hours with a hollow fiber membrane lung. The clotting times and plasma heparin levels were low, following an initial rise after going on bypass. Clots were observed in areas of the circuit where blood flow was stagnant, in particular the outlet header caps and the oxygenation volume displacer.[8]

In the current study, all animals were awake, alert, and survived 96 hours of venovenous bypass with no visible respiratory or neurologic side effects. The normal side effects of bypass, thrombocytopenia and leukopenia with a subsequent rebound, occurred. The activated clotting time after a small initial rise was within the normal range.

Postmortem examination of the circuits confirmed some of our earlier work and that of Bindslev. All high velocity flow components were clot free, indicating that Duraflo II was very effective in maintaining a strong bond with each biomaterial. Stagnant flow zones in the header caps, volume displacers and some areas of the heat exchanger were noted to have mild to moderate white thrombi. The stagnant zones may have contributed to the drop in circulating platelets. The clots found in one animal's lung may have been generated from stagnant zones within the circuit.

Duraflo II is a coating material that consists of heparin and a proprietary binding agent. The coating material is highly stable in crystalloid solution, and can be applied to virtually all of the extracorporeal surfaces and biomaterials without problems of stress cracking and swelling. No activity of factor Xa and thrombin inhibition were measured in prime samples; thus the lack of free heparin in the rinse indicates the heparin bond is stable, as does the small rise in ACT after initiating bypass.

The mechanism by which Duraflo II renders its potency is not clear. Protein deposition occurs instantaneously when blood comes in contact with a foreign surface.[9,10] The presence of surface-bound heparin may modulate protein deposition and provide a more thromboresistant surface.

Duraflow II provides an effective heparin coating in sheep during 4 days of ECMO. The coating uniformly bound to different biomaterials, which is not the case for some benzalkonium heparin coatings. The major limiting factor in the use of this or any other heparin coating is the flow velocity and distribution of flow through the circuit, as low blood flow and stagnant zones will cause the blood to clot regardless of the heparin coating. Special consideration must be taken to design artificial organs to minimize eddy currents,

stagnant or dead-end flow zones, and to maximize laminar flow areas. Proper flow distribution will minimize stagnation, platelet consumption, and potential thrombosis.

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Non-thrombogenic Hemofiltration System for Acute Renal Failure Treatment

SUN-DE TONG AND LI-CHIEN HSU

Continuous arteriovenous hemofiltration (CAVH) has become an accepted therapy for patients with acute renal failure. A major technical concern with CAVH is clotting of the hemofilter, resulting from blood–material interactions. This study compares the thromboresistance and performance characteristics of a Duraflo II heparin treated CAVH circuit with those of an untreated control circuit. The circuit consisted of a polysulfone hemofilter, tubing sets, and catheters. The heparin treatment did not change the mass transfer properties of the hemofilter. The thromboresistance of the heparin treated circuit was compared directly with that of an untreated circuit in a heparinless sheep model using bilateral circuits. The heparin treatment significantly enhanced the blood compatibility of the circuit, as indicated by the reduction in thrombus formation, prolonged work life, and superior performance in heparinless sheep. There was minimal heparin leaching from the hemofilter. A heparin treated circuit may improve the safety and effectiveness of the CAVH procedure, and offers potential for a systemic heparin protocol to be modified for patients with bleeding disorders. *ASAIO Journal* 1992; 38: M702–M706.

One of the major limitations of the use of extracorporeal devices that require contact with blood is the thromboembolic phenomena that occur at the blood–foreign material interface. Systemic heparinization usually is required during extracorporeal blood circulation to prevent thrombus formation in the circuit. However, use of systemic heparin can increase the risk of bleeding in patients with coagulation disorders or following surgery. Low dose heparinization,[1] regional heparinization,[2] and citrate anticoagulant[3] have been used in attempts to control the bleeding in patients with renal failure. Despite these efforts, clotting of the hemofilter and coagulation disorders in patients with acute renal failure continue to be major concerns during hemofiltration. One approach to minimizing blood trauma during extracorporeal blood circulation is to improve the thromboresistance of the circuit. Recent studies into heparin coating of extracorporeal devices for cardiopulmonary bypass[4] and extracorporeal membrane oxygenation[5] have shown that systemic heparin could be substantially reduced or eliminated when the entire bypass circuit was treated with Duraflo II heparin. This study compares the thromboresistance and performance characteristics of a Duraflo II heparin treated continuous arteriovenous hemofiltration (CAVH) system, with those of an untreated control system in a non-heparinized sheep. The stability of the heparin treatment in plasma, and the effect of heparin treatment on functional performance of the hemofilter, were also evaluated *in vitro*.

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Materials and Methods

Materials

The hemofiltration system consisted of a polysulfone hollow fiber hemofilter (Diafilter 20; Amicon, Beverly, MA), CAVH tubing set (Amicon), and single lumen catheters (model MC7CAVH4; Medical Components Inc., Harleysville, PA). The entire blood contact surfaces of the test CAVH system were treated with Duraflo II heparin (Bentley Labs, Baxter Healthcare Corp., Irvine, CA). The general coating procedures are similar to those described previously.[5] Coating conditions were selected to ensure that the permeability and wetting properties of the hollow fiber membrane were not altered. A control system containing the same devices was used as received. Both control and test systems were sterilized with ethylene oxide gas before testing.

Stability of Duraflo II Heparin Treatment

The stability of the heparin treatment was determined by circulating EDTA anticoagulated bovine plasma through

filters (n = 3) treated with H^3 labeled Duraflo II heparin; 200 ml bovine plasma was recirculated at 150 ml/min for 68 hr. Plasma samples were taken periodically to determine, with a scintillation counter, the amounts of heparin leached.

In Vitro *Functional Performance*

To assess the effect of Duraflo II heparin treatment on the mass transfer properties of the hemofilter, the ultrafiltration rate (UFR) of Duraflo II heparin treated hemofilters (n = 6) was measured at various blood flow rates and transmembrane pressures (TMP) using EDTA anticoagulated bovine blood. Sieving coefficients were also determined in aqueous solution or bovine blood, and results compared with those of untreated control hemofilters (n = 3).

Ex Vivo *Heparinless Ovine Evaluation*

Bilateral *ex vivo* circuits that allow simultaneous evaluation of heparin treated and untreated control CAVH systems, were used (**Figure 1**). Five sheep (average weight, 42 kg) studies were conducted without the use of systemic heparin. Two of the five heparinless studies were conducted for 6 hr, during which the animals remained anesthetized throughout the procedure. The other 3 studies were conducted for 24 hr. Each animal in the 24 hr studies was initially anesthetized during cannulation and subsequently awakened and restrained in a sling for the duration of the test period. Each animal was prepared for the CAVH procedure by surgically exposing the femoral or carotid artery and femoral or jugular vein on both sides of the animal. The test circuit (heparin treated) was connected to one side of the animal, and a control circuit to the other side.

Before cannulation, both test and control circuits were rinsed and primed per manufacturer's instructions for use. Throughout the test period, similar transmembrane pressures were maintained for both control and test hemofilters by adjusting the back pressure of the hemofilters or the head

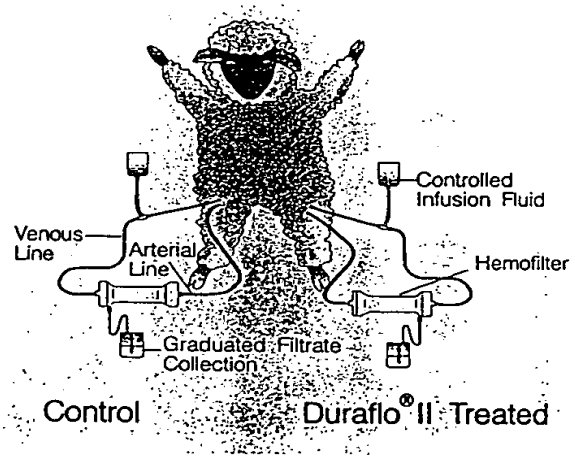

Figure 1. Schematic diagram of the bilateral CAVH circuits.

Table 1. ***In Vitro*** **Ultrafiltration Rates of Duraflo II Heparin Treated and Control Amicon Diafilter 20**

Hemofilter	Blood Flow Rate (ml/min)	TMP (mmHg)	Blood Path ΔP (mmHg)	UFR (ml/min)
Control	75	50	11 ± 2	12 ± 1
Duraflo II	75	50	11 ± 1	15 ± 1
Control	75	100	12 ± 1	24 ± 1
Duraflo II	75	100	11 ± 1	24 ± 1
Control	100	50	14 ± 2	12 ± 3
Duraflo II	100	50	13 ± 2	15 ± 1
Control	100	100	13 ± 2	25 ± 4
Duraflo II	100	100	13 ± 2	28 ± 1
Control	200	50	25 ± 4	14 ± 3
Duraflo II	200	50	24 ± 3	17 ± 2
Control	200	100	26 ± 4	31 ± 3
Duraflo II	200	100	24 ± 3	35 ± 2

height of the ultrafiltrate collection containers. The fluid balance of the animal was maintained by reinfusing the collected ultrafiltrates. Transmembrane pressures and UFR were closely monitored throughout the test period. Activated clotting time (ACT), blood flow rate, total cell count, blood chemistry, and hematocrit were determined periodically. The circuit was removed from the animal either at the end of the test period, or after the filter failed to function, as indicated by the lack of ultrafiltrate at a given TMP. The circuit was then gently and thoroughly rinsed with saline and examined macroscopically for thrombus formation.

Results

Average ultrafiltration rates of the heparin treated and untreated control filters determined *in vitro* at various blood flow rates and TMPs are shown in **Table 1**. Sieving coefficients for albumin, creatinine, sodium, BUN, phosphorus, insulin, and vitamin B_{12} are shown in **Table 2**. No differences in any of the parameters measured were noted between the heparin treated and untreated hemofilters.

The amount of heparin leached from the Duraflo II treated hemofilter into the circulating plasma, expressed as a percentage of the total Duraflo II heparin content on the device, is shown in **Figure 2**. The total amount of Duraflo II heparin contained in the hemofilter is equivalent to about 800 USP

Table 2. ***In Vitro*** **Sieving Coefficients for Duraflo II Heparin Treated and Control Amicon Diafilter 20**

Solute	Control (n = 3)	Test (n = 6)
Albumin	0	0
Creatinine (MW = 113)	0.88 ± 0.04	0.97 ± 0.06
Sodium	0.99 ± 0.06	1.00 ± 0.06
BUN (MW = 60)	1.02 ± 0.17	0.92 ± 0.19
Phosphorous	0.98 ± 0.10	0.99 ± 0.06
Insulin* (MW = 5000)	1.02 ± 0.01	1.00 ± 0.02
B-12* (MW 1355)	1.00 ± 0.01	1.00 ± 0.01

* Tested in aqueous solution.

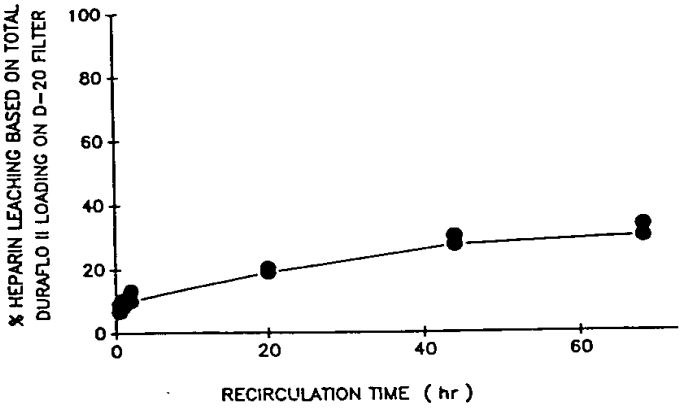

Figure 2. Mean values of heparin detected in plasma, expressed as percent of total heparin contained in the hemofilter.

units. The amount of heparin detected in circulating plasma at 1 hr, 20 hr, and 68 hr was 60 units, 140 units, and 220 units, respectively.

The relative thromboresistance and performance of the heparin treated and control CAVH circuits were determined in an *ex vivo* heparinless sheep model using bilateral circuits. To provide an identical test condition for both circuits, the TMPs of the treated and control hemofilters were maintained at similar values. Plots of TMP *versus* time for each 24 hr study are shown in **Figure 3A–3C**. The measured UFRs at similar TMPs, expressed as a function of CAVH time for each 24 hr heparinless experiment, are shown in **Figure 4**. In all cases, the control filters exhibited a rapid reduction in UFR shortly after the initiation of CAVH. The UFR continued to decrease with time. All control filters completely clotted off in less than 6 hr of CAVH in heparinless sheep. The clotted circuit was subsequently removed from the animal to allow evaluation of the treated circuit to continue. The corresponding heparin treated filters exhibited a smaller initial decrease in UFR, and remained functional throughout the test periods, after which the test circuits were electively removed. Clearance rates of the treated and untreated control filters closely followed those of UFR as shown in **Figure 5**. No obvious thrombocytopenia, leukopenia, or leukocytosis were noted throughout the testing periods, nor were changes in activated clotting time. **Figure 6** shows the typical appearance of treated and untreated hemofilters after heparinless CAVH in sheep. Massive clots were noted on the control hemofilter, whereas the heparin treated filter was relatively free of thrombi.

Discussion

Heparin coating of blood contact surfaces, until very recently, has been limited to relatively simple devices, such as catheters and shunts. Recent developments in heparin coated cardiopulmonary bypass circuits represent a major advance toward improved thromboresistance of artificial devices.[6] One key requirement for heparin coating of a hemofilter or hemodialyzer is that the coating should not interfere with the mass transfer properties of the device. Heparin coating techniques that involve the use of polymeric binders, or modification of membrane substrate, may have a detrimental effect on the convective or diffusive transport processes of the membrane. To avoid the negative impact

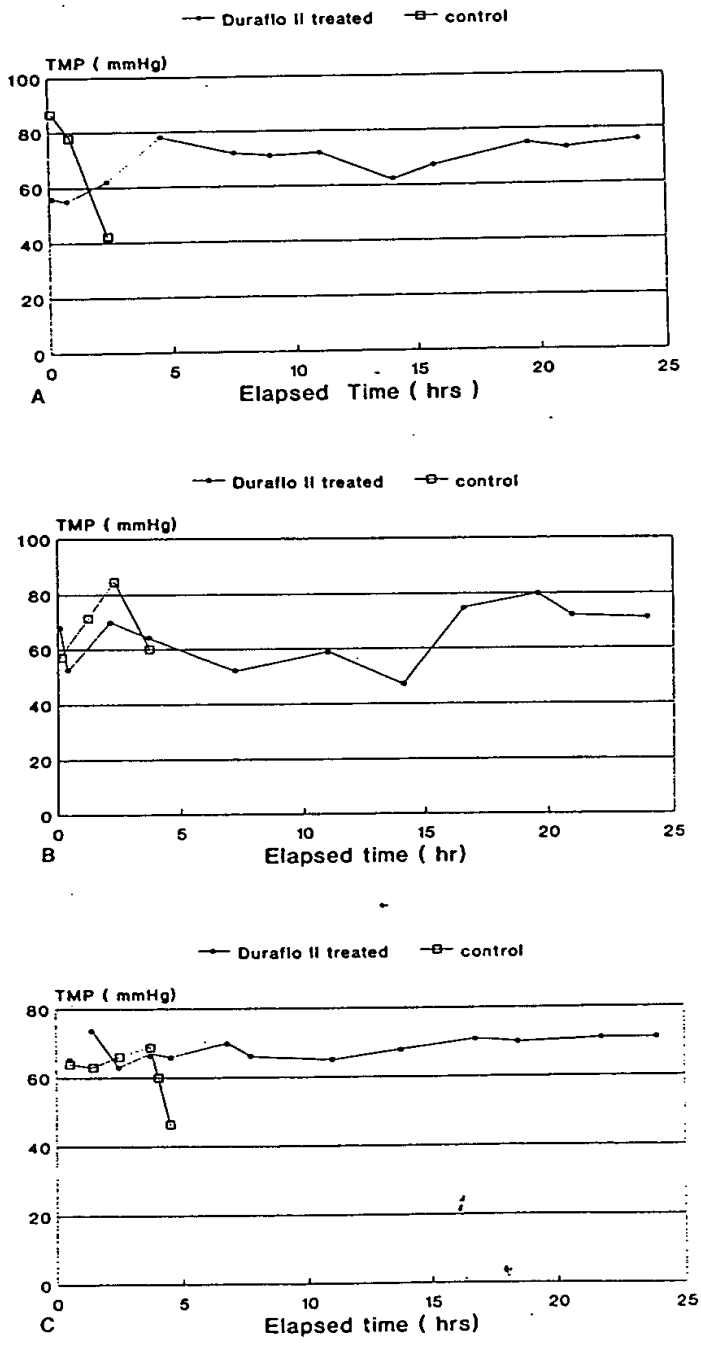

Figure 3. Time *vs.* transmembrane pressure of the Duraflo II heparin treated and control CAVH circuits. (A) Test 1: (B) Test 2: (C) Test 3.

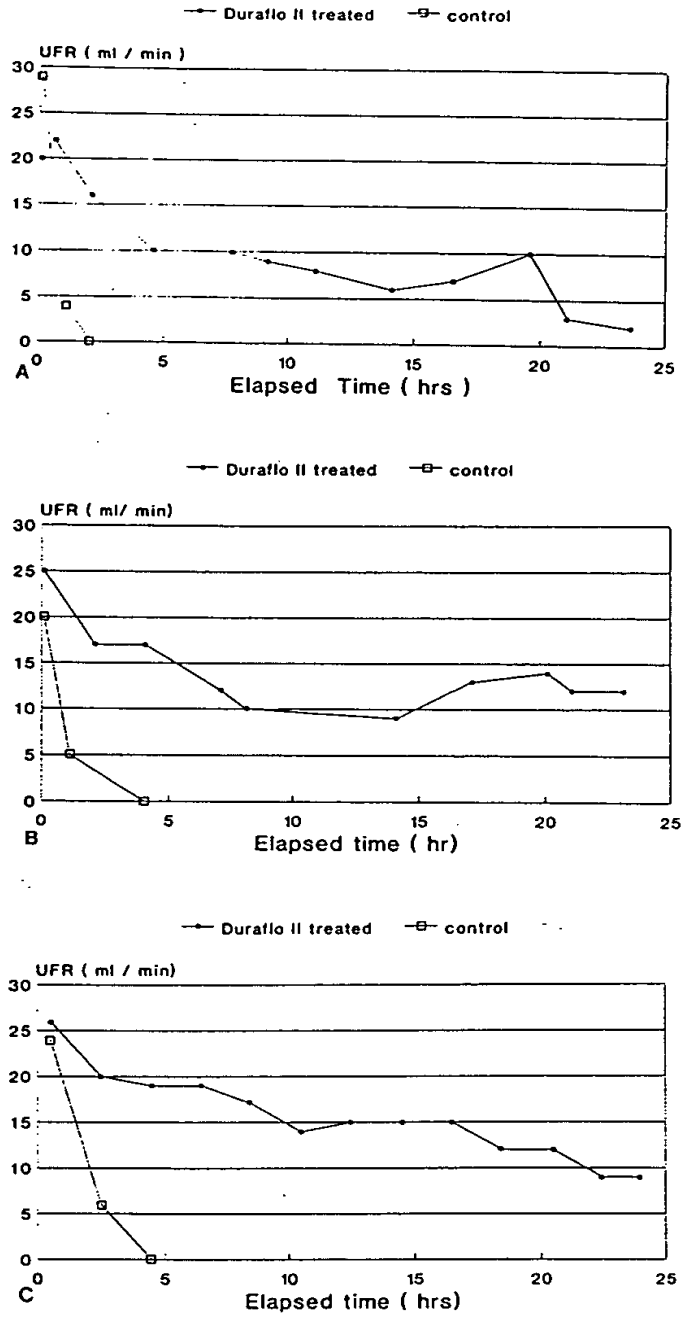

Figure 4. Time *vs.* ultrafiltration rate of the Duraflo II treated and control CAVH circuits. (A) Test: (B) Test 2: (C) Test 3.

on mass transfer properties due to heparin coating, Arakawa et al developed a hydrophilic membrane, along with a heparin coated catheter, tubing, and header to enhance the thromboresistance of an ultrafiltration system.[7] In the current study, Duraflo II heparin treatment was applied to the various components of the CAVH system. *In vitro* performance results (Tables 1 and 2) comparing a Duraflo II heparin treated hemofilter and untreated control filter clearly indicate that the mass transfer properties of the polysulfone membrane are not affected by the heparin treatment. Simila

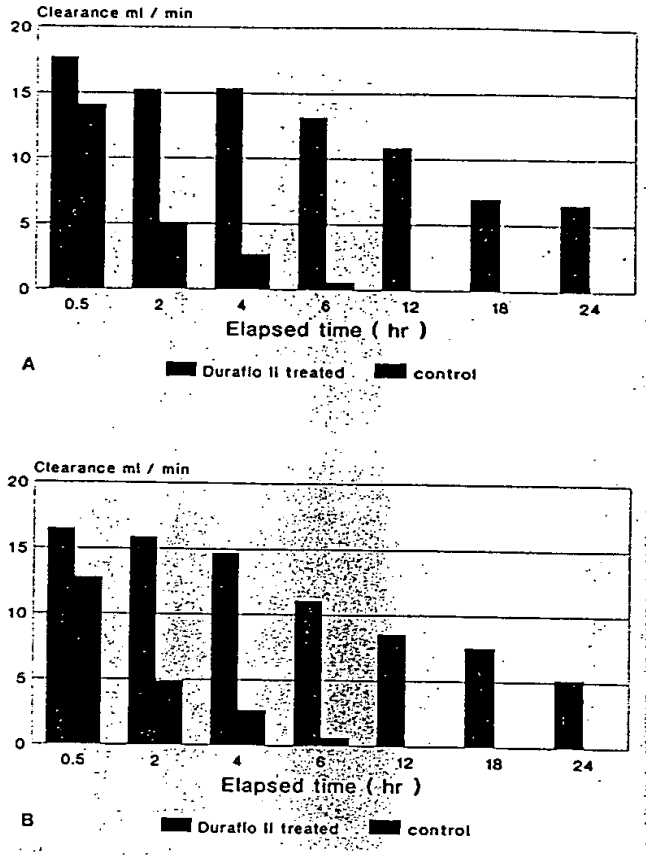

Figure 5. Average clearance rates of the Duraflo II treated and control CAVH circuits. (A) BUN. (B) Creatinine.

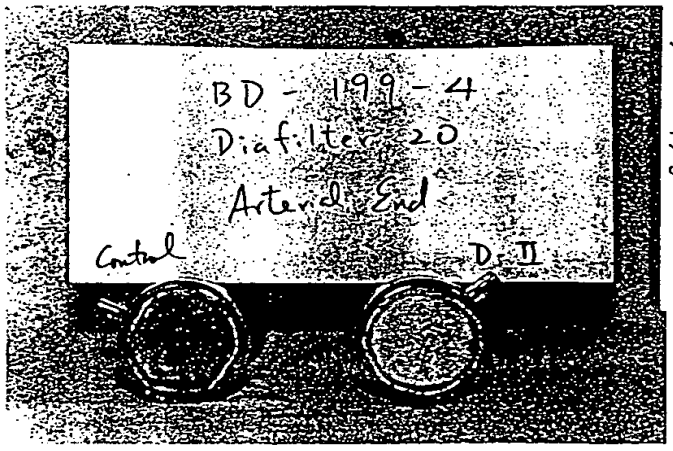

Figure 6. Typical appearance of Duraflo II heparin treated and control hemofilters after heparinless CAVH in sheep.

ultrafiltration rates at a given TMP between heparin treated and control hemofilters suggest that the wetting properties of the polysulfone membrane are not altered by heparin treatment.

Stability of the Duraflo II heparin coating system on an arterial blood filter has been demonstrated previously.[8] In the current study, radioactively labeled Duraflo II heparin was used to determine the stability of the coating on a polysulfone hemofilter in plasma. The results indicated that the amount of heparin leached into circulating plasma was insignificant, and was far less than the amount suggested as necessary for a heparinized hydrophilic polymer.[9] The *in vitro* results are consistent with *ex vivo* animal studies that showed no changes in ACT.

The thromboresistance of the Duraflo II heparin treated CAVH system was compared directly with that of an untreated system in a heparinless *ex vivo* sheep model. No detailed hematologic study was performed in the current study, since blood flowed through both treated and untreated circuits. However, the experimental design does provide a condition that allows for direct assessment of the relative performance and thromboresistance of the two circuits, because blood having identical thrombotic and hemostatic status circulates through both systems. Under this condition, the Duraflo II heparin treated system showed significantly and consistently higher ultrafiltration rates, relative to those of the untreated system. The usable life of the heparin treated system also was substantially prolonged, compared with that of the untreated system. The enhanced performance and prolonged usable life of the heparin treated circuit are a result of reduced thrombus formation in the circuit. The improved blood compatibility of the heparin treated circuit cannot be attributed to heparin release from the circuit, because only an insignificant amount of heparin was leachable. Previous study has shown that immobilized heparin was many times more effective than corresponding circulating heparin.[10] The mechanism by which the immobilized heparin functions still is not clearly understood. It is possible that heparin placed at the sites of blood activation (foreign surfaces) modulates the types of protein adsorption that may be ultimately responsible for long-term blood compatibility. Immobilized heparin also may be more effective in inhibiting the contact activation system.

Potential benefits resulting from the use of Duraflo II heparin treated extracorporeal circuits have been demonstrated clinically. Reductions in activation of the coagulation system[11] and inflammatory responses,[12] as well as reductions in blood loss and blood transfusion requirements[4] in patients undergoing routine cardiopulmonary bypass, have been reported. Totally heparinless CPB also has been performed in a patient with multiple trauma and hypothermia.[13] It appears that similar benefits may be expected from the heparin treated CAVH circuit.

The efficacy of CAVH therapy depends, in large part, on clearances continuously maintained over a long period of time. The results in this study suggest that the Duraflo II heparin treatment may enhance the safety and efficacy of the CAVH procedure. The heparin treated CAVH system may be useful for patients who are less tolerant of systemic anticoagulants, and may allow the systemic heparin protocol to be modified so as to prevent filter clots in patients with bleeding disorders.

Acknowledgments

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Reduction of fibrinogen adsorption on PEG-coated polystyrene surfaces

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Reduction of protein adsorption by coating surfaces with polyethylene glycol (PEG) is well documented. The present work has four goals related to these previous studies: first, to develop chemistry providing densely packed, covalently bound PEG on polystyrene (PS); second, to determine the ability of these modified surfaces to reject fibrinogen; third, to compare the protein-rejecting ability of branched and linear PEGs; and fourth, to examine the utility of an ELISA-type procedure for measuring protein adsorption. It was found that PEG-epoxide could be readily coupled to amine groups of poly(ethylene imine) (PEI), which had been preadsorbed onto an oxidized PS surface. The PEG groups on branched PEGs appear to act as an excluded volume to repel proteins, similar to arguments previously raised for linear PEGs. The results of protein adsorption studies showed that fibrinogen adsorption is significantly reduced by coating polystyrene with either linear or branched PEGs of 1500 to 20,000 in molecular weight. The ELISA technique was found to be equivalent in sensitivity to radiolabeled fibrinogen for estimating adsorption levels. It is expected that PEG-coated PS will have much utility in a variety of biomedical applications.

INTRODUCTION

There has been much recent interest in use of PEG coatings as a means of reducing protein adsorption on a variety of surfaces.[1–4] We have a particular interest in applying this technique to polystyrene (PS) surfaces because of the frequent use of PS lab ware (such as microtiter plates) in biochemical assays where nonspecific protein adsorption can produce misleading results.[5] One approach is to physically adsorb PEG-based surfactants onto the surface.[4,6,7] Since there is a strong possibility that adsorbed material may be desorbed (especially in the presence of proteins that compete for surface sites), we have concentrated on more permanent attachment to PS.

Additionally,[8] we have developed a simple, ELISA-type protein assay for this study. Advantages of this technique are that there is no need to work with radioactive material and that the rapid-reading capability of the ELISA

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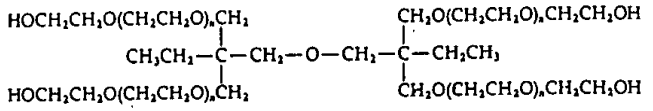


Scheme 1

technique is made available. Hence, a secondary goal of the present work was to compare results obtained with the ELISA method and with the conventional ^{125}I procedure.

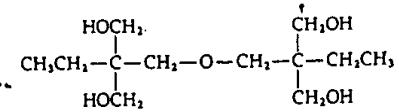
Finally, we have included some tetra-branched PEGs (Scheme 1) in our study, first, to determine whether branching could give improved coverage of the surface, and second, to gain insight into the mechanism by which PEGs act in reducing protein adsorption. The branched PEGs were made by ethoxylation of di-(trimethylol)propane (Scheme 2).

MATERIALS AND METHODS

Human fibrinogen was purchased from Kabi, Sweden (Grade L) and iodinated as previously described.[9-11] Linear PEG 1500 and 4000 and branched PEG 1700, 5000 and 14,000 were obtained from Berol Nobel and linear PEG 20,000 was obtained from Fluka. UV spectroscopy was performed on a Dynatech MR 600, HPLC analyses were performed with a Waters Ultrahydrogel 250 column (crosslinked methacrylate gel packing), NMR spectroscopy was performed on an IBM-Brucker AM-200, and XPS spectroscopy was performed on a Perkin-Elmer 5400 (Alabama) or a Surface Science Laboratories SSX-100 (Washington). A TM Analytic, Model 1185 gamma counter was used for measuring ^{125}I concentrations.

Preparation of glycidyl ethers of PEG

PEG glycidyl ethers were prepared by one-step reaction of PEG with epichlorohydrin in the presence of solid sodium hydroxide. The method used is a slight modification of the procedure described by Gu, Ikeda, and Okohara for preparation of low-molecular-weight glycol diglycidyl ethers.[12] In a typical procedure, PEG 5000 (25 g, 1 equiv. of terminal OH groups) was added as a powder to a mixture of epichlorohydrin (6.7 g, 15 equiv.), BHT (0.03g), sodium hydroxide (1.2 g, 3 equiv.) and water (0.15 g or 0.125 g per g NaOH) and heated to 61°C for 10 h. Water (100 mL) and dichloromethane (200 mL) were added, and solid NaH_2PO_4 was added to adjust the pH of the water



Scheme 2

layer to 7. After separation, the organic layer was washed twice with 100 mL water. The organic layer was separated and the solvent was removed by rotoevaporation (90°,1 h). The product obtained was dissolved in 80 mL dichloromethane and added gradually to 900 mL cold diethyl ether to precipitate the product, which was filtered off. This precipitation process was repeated, and the solid product dried under vacuum at room temperature. Yield 80%.

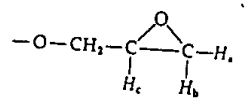
For lower-molecular-weight PEGs a 5-to-1 excess of epichlorohydrin was used. The low-molecular-weight liquid epoxide (such as that from b-PEG 1700) was purified by first dissolving the crude product (from methylene chloride extraction, above) in 150 mL distilled water and extracting yellow impurities with 7 mL dichloromethane, which is discarded. The remainder of epoxide is extracted from the water layer with three 75-mL portions of dichloromethane. Most of the dichloromethane was distilled off, and the resulting concentrated solution of epoxide applied to a silica gel column (200 g, "Merck 60," 230–400 mesh, Aldrich). Impurities were removed by elution with 1000 mL dichloromethane, and the product was eluted with 1400 mL of methanol. The methanol was removed by rotoevaporation to give the final pure product. Yield 75%.

The reaction was monitored by NMR and titration of epoxide groups following the method of Jay.[13] A few drops of crystal violet indicator (from a solution of 0.1 g crystal violet in 100 mL acetic acid) is added to a solution of 100 g tetraethylammonium bromide in 400 mL acetic acid, and 0.1*M* $HClO_4$ in acetic acid is added dropwise until the color changes. The tetraethylammonium bromide solution (10 mL) is mixed with a solution of PEG-epoxide in chloroform (0.5–1.0 meq. epoxide in 10 mL solution). A few drops of the crystal violet indicator solution is added and tritration is made with 0.1*M* $HClO_4$ until a blue color appears. Addition of $HClO_4$ to the tetraethylammonium bromide solution generates HBr which is rapidly consumed by reaction with the oxirane ring. The concentration of epoxide groups can be calculated from the expression

$$\text{meq. epoxide/g} = [(a - b)n]/c$$

where a and b are number of milliliters consumed by sample and blind respectively, n is molarity of the $HClO_4$ solution, and c is number of grams of epoxide compound. The epoxides made here gave 100–110% conversion to epoxy groups.

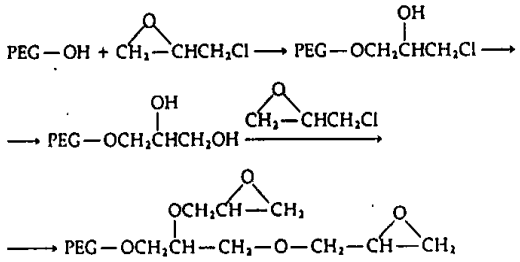
Proton NMR spectra of the glycidyl ethers in d_6-dimethylsulfoxide show characteristic epoxide absorbances at 2.55 ppm (H_a), 2.72 ppm (H_b), and 3.09 ppm (H_c):



The large backbone (CH_2CH_2O) absorbance is centered at 3.5 ppm. No hydroxyl absorbances were observed at 4.57 ppm, thus indicating complete con-

version of hydroxyl groups to glycidyl ethers.[14] Comparison of backbone areas to the ring areas gives slightly greater than 100% conversion in all cases, as also shown by titration.

The measurement of >100% conversion (i.e., slightly more than two epoxide groups are introduced per PEG molecule) may be due to a side reaction proceeding via a glycerol monoether of PEG which can react with two moles of epichlorohydrin:



PEG-coated PS

PEG immobilization was performed by reaction of PEG epoxide with aminated PS, which was prepared by a two-step process of surface oxidation followed by adsorption of poly(ethylene imine) (PEI) according to a procedure published by Kiss, Golander, and Eriksson.[15]

Test tubes and microplates were washed in 70% ethanol for 3 min in an ultrasonic bath (Branson 220) and then permitted to air dry. The dry objects were oxidized with 2 g/L $KMnO_4$ in concentrated sulfuric acid for 30 s and thoroughly washed with distilled and deionized water. A 0.05 M carbonate buffer, pH 9.5, containing 3% PEI (Polymin SN-BASF; ratio of primary to secondary to tertiary nitrogen, 1:2:1) was added to each test tube or well, and the solutions were permitted to sit for 30 min. The tubes or plates were rinsed with water, filled with a solution containing 10% PEG-epoxide in carbonate buffer, and reacted at 37°C for 3 h. The objects were then thoroughly rinsed with water and dried in a desiccator.

Preparation of radiolabelled fibrinogen

Human fibrinogen was labelled with ^{125}I by the iodine-monochloride method of MacFarlane,[9] modified by Helcamp[10] and Horbett.[11] Briefly, one mCi of $Na^{125}I$ (Amersham) was added to 0.5 mL of 0.4*M* borate and 0.32*M* sodium chloride at pH 7.75. This solution was mixed with 0.5 mL of cold iodine monochloride in 2*M* sodium chloride. This solution was then added to 0.5 mL of fibrinogen in borate buffer and mixed by gentle repipetting. After 20–30 min of reaction at room temperature, the free ^{125}I was separated from the

labeled fibrinogen by gel chromatography (BioGel P-4, Biorad) at room temperature using citrate-phosphate buffered saline (CPBS, 0.01*M* citric acid, 0.01*M* sodium phosphate monobasic, 0.12*M* sodium chloride, 0.02% sodium azide, pH 7.4) as the mobile phase. The labeled protein was collected, stored at −70°C and used within 2 weeks of preparation.

Measurement of fibrinogen adsorption (^{125}I)

Prior to adsorption, the surfaces were rehydrated in CPBS (containing 0.01*M* NaI) overnight at 4°C, then the buffer was replaced with fresh degassed buffer, and the samples were equilibrated at 37°C for about 2 h. Adsorption was initiated by adding a sufficient amount of radioactive fibrinogen solution so that the final concentration of the fibrinogen in the tubes was 0.2 mg/mL. Adsorption was allowed to take place for 2 h at 37°C, after which the tubes were rinsed by the dilution–displacement method, which washes away the unbound or loosely bound fibrinogen while avoiding exposure of the surface to air. After rinsing, the radioactivity of the tubes was measured by gamma counting.

Measurement of fibrinogen adsorption by ELISA

PEG-coated test tubes were rehydrated overnight at 4°C in CPBS. The buffer was replaced with 2 mL fresh, degassed CPBS, and the samples were equilibrated for 2 h at 37°C. Human fibrinogen (0.4 mg) was added and mixed by gentle repipetting. After 2 h at 37°C, each sample was rinsed by dilution-displacement with 100 mL CPBS. Two milliliters of a 0.1*M* phosphate buffer, pH 7.0 (containing 1.0*M* NaCl and 0.2% Tween 20), containing 0.01 mg/mL peroxidase-conjugated goat immunoglobulin to human fibrinogen and 0.05 mg/mL rabbit immunoglobulin to human fibrinogen (Dakopatts, Denmark) was added to each test tube. After 1 h at room temperature the test tubes were rinsed extensively with water. Color was developed by adding 2 mL of a solution containing 0.67 mg/mL 1,2-phenylenediamine dihydrochloride (Dakopatts) in 0.01% hydrogen peroxide (Merck). The reaction was terminated after 5 min with 2 mL of 1*M* sulphuric acid and the absorbance read at 490 nm. The color produced is proportional to the amount of adsorbed fibrinogen. Adsorption of fibrinogen on the microplates was measured in the same way but with a total amount of 250 μL of solution per well.

RESULTS AND DISCUSSION

PEG-coupling chemistry

Direct evidence that the chemistry employed here results in attachment of PEG to the PS surface can be derived from x-ray photoelectron spectroscopy

(XPS). As expected from the work of Gölander and Ericksson[15,16] (with polyethylene) XPS shows that $KMnO_4/H_2SO_4$ oxidaton of the PS surface produces a variety of oxidized carbon and sulfur groups including aldehyde, alcohol, carboxylate, ether, and sulfate. Oxidation produces a reduction in %C, a large increase in %O, and the appearance of a small sulfur peak Table I. The C1s XPS spectrum of oxidized PS reveals two new peaks at 288.9 (relative area 1) and 286.5 eV (relative area 2) in addition to the —C—C— peak of PS at 284.7 eV (relative area 4) (Fig. 1). The peak at 286.5 eV is consistent with ether, alcohol, and peroxide groups, and the peak at 288.9 eV is consistent with carboxyl and aldehyde groups. The presence of sulfur-containing groups is revealed by measurement of 1.8–2.6% sulfur in the XPS spectra of several samples.

Adsorption of the branched PEI to this oxidized surface gives a surface containing 10–11% nitrogen, reduced %O, and altered carbon type (Table I and Figure 1). Previous work has shown that adsorption of this branched, high MW PEI gives a large number of irreversibly bound, primary amino groups on the surface.[15,16]

In the final step, coupling with PEG reduces the nitrogen percentage to 3–6% (presumably because PEG covers the PEI), increases %O, and gives the typical XPS carbon spectrum expected for a surface containing a large amount of coupled PEG; i.e., the C1s region of the spectrum contains a large —C—O— peak at 286.5 eV.[15–18] Spectra for the different, surface-bound PEGs were very similar, with essentially identical ratios of —C—O— to —C—C— carbon (typically around eight). In other words, XPS spectra show PEG attachment and reveal no difference between small and large or linear and branched PEGs. The lack of variation of XPS spectra for the different molecular weights is consistent with a densely packed PEG coating on the surface. If the small PEG molecules, but not the large ones, were far enough apart to flatten out on the surface in the dehydrated state necessary for XPS measurements then one would expect the larger PEGs to give larger ether peaks.

Although the present experiments do not permit direct measurement of the surface density of PEGs, the observed —C—O—/—C—C— ratios in the C1s XPS spectra are similar to those observed previously for polyethylene,[15,16] mica,[17] and glass[18] surfaces on which PEG density varied from 190–350 Å^2/PEG.

The PEG-epoxides used in this study are either bifunctional (for linear PEGs) or tetrafunctional (for branched PEGs), and thus the possibility of multipoint surface attachment (or "bridge" formation) must be considered. Three pieces of indirect evidence indicate that bridge formation is not occurring. First, as indicated by ESCA spectra and protein adsorption results (below), the coatings obtained are dense, with PEGs crowded together more closely than their solution exclusion volumes.[15–18] In this environment the individual molecules will tend not to bend over and contact the surface again. Secondly, the attachment process is conducted with a huge excess (see Experimental) of active PEG relative to surface amine groups, a factor that favors attachment of unbound PEGs relative to bound PEGs. Finally, we note that two previous studies have shown that difunctional, linear PEGs bound to surfaces have many active groups remaining for coupling of other molecules, and thus these PEGs are not bridged.[19,20]

TABLE I
Results of XPS Studies of a Series of Polystyrene (PS) Samples.[a]

Sample	%C	%O	%N	%S
Untreated PS	96–98	1–3	tr	—
Oxidized PS	62–63	33	<1	1.8–2.6
PEI adsorbed PS	71–74	15	10–11	0.7–2.0
PEG coupled PS	64–69	25–29	3–6	tr

[a]All measurements were performed on a minimum of two samples. Trace (tr) amounts of Si, Cl, Zn, and Na were observed on most samples.

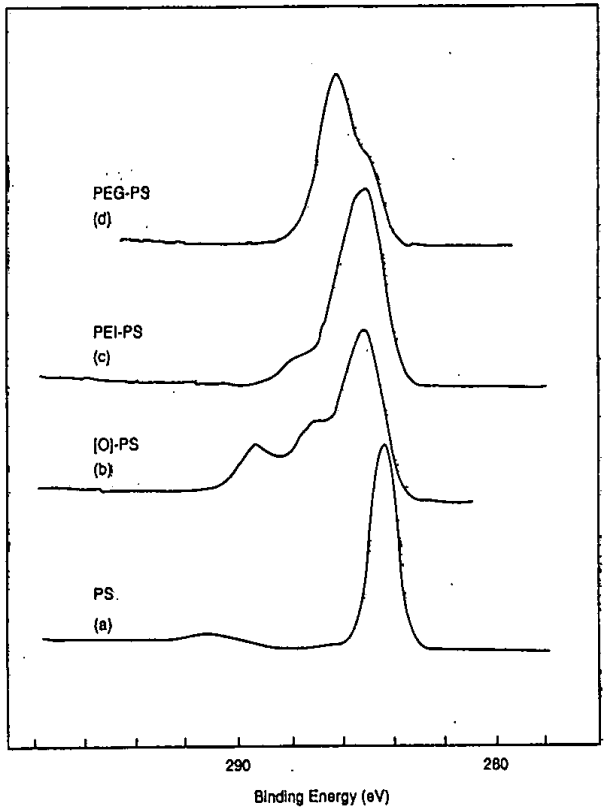


Figure 1. C1s XPS spectra for: (a) untreated PS, (b) oxidized PS, (c) oxidized PS treated with branched PEI, and (d) PEI-PS treated with epoxy-PEG.

These PEG coatings were stable for extended periods upon dry storage as shown by independence of ESCA spectra and protein adsorption (below) with storage time. Similarly, exhaustive aqueous washing produced no observable effect on the PEG coatings.

Comparison of methods for measuring fibrinogen adsorption

As can be seen from Table II the PEG coatings reduce fibrinogen adsorption by generally greater than 90% for all molecular weights and shapes. Control experiments with unmodified PEG showed no protein rejection effect, because (as shown by ESCA) the polymer is removed from the surface by routine washing. The two methods used to determine protein adsorption give very similar patterns (within experimental error) thus confirming the validity of the ELISA method.[8] As described in the introductory comments, the ELISA method avoids the need for radioactive materials and it permits rapid, automated washing and reading of samples. One disadvantage of the method is that it does not give an absolute amount of protein on the surface, but rather provides relative amounts of adsorption. Similarly, the ELISA technique responds only to the surface layer and will not detect multilayers of adsorbed protein.

In these experiments we used both PS test tubes (coatings on inside only) and PS microtiter plates (coatings in wells). Earlier experiments with PS slides gave poorer reproducibility than did these sample configurations, presumably because the flat slides were easier to damage physically during coating, washing and assaying. Consistent with this hypothesis, PS tubes give lower degrees of fibrinogen adsorption than do the more "open" microtiter plates (Table II).

Effects of PEG MW and branching on fibrinogen adsorption

Table II also shows no MW dependence on PEG fibrinogen-rejecting ability. Examination of earlier studies in which a MW dependence was noted,[21-24]

TABLE II
Results of Fibrinogen Adsorption Measurements (Triplicate) by the ELISA and I^{125} techniques for linear PEGs (l-PEG) and Branched PEGs (b-PEG) on PS

Sample	Protein ads. (ELISA)		Protein ads. (^{125}I)	
	PS tube (%)	PS plate (%)	PS tube (%)	PS tube (ng/cm^2)
Untreated	100 ± 11	100 ± 5	100 ± 1	112 ± 1
l-PEG 1500	1 ± 5	—	5 ± 1	6 ± 1
l-PEG 4000	3 ± 2	7 ± 15	4 ± 1	4 ± 1
l-PEG 20000	5 ± 2	12 ± 9	4 ± 1	4 ± 1
b-PEG 1700	4 ± 3	—	5 ± 1	6 ± 1
b-PEG 5000	1 ± 5	8 ± 4	5 ± 1	6 ± 1
b-PEG 14000	6 ± 7	13 ± 16	10 ± 5	11 ± 6

shows that the MW dependence is most dramatic for small PEGs with MWs below 1000, with a plateau being reached by approximately 2000 and little change above 1500. Thus it is perhaps not surprising that our study with PEG MWs no smaller than 1500 shows no MW dependence. The density of the PEG coating also probably plays a role. For example, Sun et al. studied fibrinogen adsorption on Silastic, which was coated with copolymers of PEG methacrylate (PEG-MA) and hydroxyethyl methacrylate (HEMA),[23] and noted that there is a molecular weight dependence of fibrinogen rejecting ability at low PEG-MA/HEMA ratios (where PEGs are loosely packed) but not at high PEG-MA/HEMA ratios (where PEGs are tightly packed). Similarly, Merrill and coworkers observed protein adsorption and thrombogenicity for low-molecular-weight PEG coatings, but not high molecular weight and concluded that the underlying surface was exposed for the low-molecular-weight PEGs.[25] Presumably if the coatings had been dense enough, only PEG would have been exposed to the surrounding medium even for the low-molecular-weight material.

Thus in our own study, we feel the lack of dependence of fibrinogen adsorption on linear or branched PEG molecular weight is the result of two effects: we prepared dense coatings and we studied a molecular weight range where the smallest molecular weight was already at or beyond the region where protein adsorption begins to level out. Alternatively, it has been proposed that PEG coatings are effective because the PEG–water interface has a low interfacial free energy.[4] If this factor were dominant, a dependence on PEG molecular weight would not be expected.

Table II also shows the lack of an observed effect of PEG branching on protein adsorption. This result is of interest for two reasons: First, intuitively one might expect that a branched PEG would cover the surface better by having its branches more efficiently cover bare spots on the surface. Apparently, however, the PEGs are tightly packed on the surfaces in all cases so that there are few bare spots. The lack of variation of XPS spectra for the different PEGs supports this conclusion.

Secondly, these results comparing branched and linear PEGs are pertinent to the theories that have been advanced to explain the mechanism by which PEG rejects proteins. According to one interpretation,[1-4,21] PEGs are unusually mobile polymers which provide insufficient contact time between protein and polymer necessary for adsorption. Also, Nagaoka and coworkers have suggested that small PEGs are less mobile than large ones.[21] Thus one could hypothesize that the smaller branched PEGs would have less mobile, short arms and thus would show less protein rejecting ability. The equal effectiveness of the branched PEGs, Table II, is counter to this hypothesis.

An alternative theory is that surface-bound PEGs act to reject proteins because PEGs have a large exclusion volume and compression of the polyether chain upon protein adsorption produces a large decrease in entropy.[1-4,21] Since the branched PEGs have fewer degrees of freedom than do the linear PEGs, one might therefore expect the branched PEGs to be less effective at protein rejection. The data in Table II, however, show no difference between linear and branched PEGs. Probably the entropic contribution of a

single branch point in a large, heavily hydrated molecule is too small to be significant.

In support of this conclusion, size exclusion chromatography (aqueous eluent) of the PEGs used in this study shows that the branched PEGs fall on a plot of log molecular weight vs. retention volume derived for linear PEGs. Thus branching produces no change in PEG exclusion volume in water. This equality of exclusion volume and protein rejecting ability for branched and linear PEGs, in our opinion, strongly supports the excluded-volume or entropic-repulsion theory of protein repulsion. Even though the average molecular weight of the PEG "arms" in a 1700 MW branched PEG is only about 400 g/mol, the arms must act in a coordinated fashion to trap water inside their excluded volume in the same way a linear, random coil PEG of 1700 MW would bind water. Immobilized linear and branched PEGs act as largely aqueous, elastic spheres that resist protein adsorption because of the accompanying compression of the sphere (entropically disfavored) or water loss (energetically disfavored). Alternatively as discussed above, the concept that PEG coatings present a surface of low interfacial free energy is also consistent with a lack of dependence on PEG branching.

There has been much recent discussion of fibrinogen activation and *enhanced* thrombogenicity caused by some PEG-coated surfaces.[25-27] The present results lead us to agree with Merrill and coworkers[25] who have concluded that the fibrinogen activation and thrombogenicity seen with some PEG-coated surfaces is the result of cooperative interaction of protein with PEG and surface sites exposed by insufficient density of the PEG coatings.

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Effect of local hemostatics on bone induction in rats: A comparative study of bone wax, fibrin–collagen paste, and bioerodible polyorthoester with and without gentamicin

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Local hemostatics for osseous tissue should preferably be absorbable and biocompatible and should not inhibit osteogenesis. The tissue response and effect on demineralized bone-induced heterotopic osteogenesis in the abdominal muscle of 120 male Wistar rats by different local hemostatics were evaluated by light microscopy and ^{85}Sr uptake analyses. Nonabsorbable bone wax of 88% beeswax and absorbable bovine fibrin–collagen paste both significantly inhibited osteoinduction, whereas a bioerodible polyorthoester drug delivery system with or without 4% gentamicin did not. Bone wax was not absorbed and induced a chronic foreign body reaction. Fibrin–collagen paste induced less inflammation with numerous monocytes and macrophages with engulfed material. Bioerodible polyorthoester caused a very moderate tissue reaction and was mostly resorbed at week 4.

INTRODUCTION

In bone surgery, nonabsorbable bone wax[1,2] of 88% beeswax and 12% isopropylpalmitat is being used for local hemostasis.[3,4] Bone wax may, however, produce a chronic inflammation with foreign-body reaction,[5] retard bone healing,[6] predispose for infections,[7,8] impair bacterial clearance,[9] and cause wax embolization.[10]

These complications have spurred the development of absorbable local hemostatics, like fibrin,[11] oxidized cellulose,[12] gelatin sponge,[13] microcrystalline collagen,[14] fibrin sealant,[15] fibrin–collagen paste,[16] fibrillar collagen,[17] and bioerodible polyorthoester sustained drug release systems,[18] which may be formulated with physical properties similar to that of bone wax, i.e., soft and moldable, and can be used as a local hemostatic in the same way.[19]

The purpose of the present study was to investigate host–tissue response and effect on demineralized bone-induced heterotopic osteogenesis by three

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MEDICAL DEVICE DESIGN - A SYSTEMS APPROACH: CENTRAL VENOUS CATHETERS

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ABSTRACT

Though biocompatibility and thromboresistance are keys to the success of medical devices, the design requirements must also address mechanical properties, durability, and easy use by the physician. Devices such as central venous catheters are also used on a daily basis by nurses and the patient and must be easy to use in both the nursing and home setting. A systems approach to the design of central venous catheters will be described. For example, the following properties can affect the success of blood contacting catheters: mechanical properties of catheter including longitudinal stiffness, kink resistance, and burst strength; surface texture including roughness, pits, and fissures; degradation which can result in toxic leachables and loss of mechanical properties; lubricity; manufacturing and sterilization methods which can result in changes in physical and mechanical properties; insertion technique into the blood vessel; size of the catheter in relation to the blood vessel; flow conditions over the catheter; duration of catheterization; nursing protocols; and patient health and medication. Infection is a constant threat with catheters that pierce the skin and can result from bacteria originating at the skin that migrate along the catheter. Approaches for dealing with the many types of potential complications will be discussed, including the use of *Controlled Environment Devices* to improve the function of central venous catheters, with particular emphasis on anticoagulant releasing coatings to reduce thrombotic complications.

KEYWORDS: Biomaterials; Central Venous Catheters; Polymers, Polyurethanes; Controlled Drug Delivery; Heparin Release Coatings; Design

1. INTRODUCTION

There are approximately 2.5 million central venous catheters used each year. These catheters were introduced in the mid 1970's, though innovation in these devices has been

slow. These catheters are used for introducing drugs to the body that require large doses or rapid dilution in a large volume of blood in order to prevent damage that would occur if infused in smaller blood vessels. These systems are used for chemotherapy for cancer patients, hyperalimentation for patients unable to digest food or malnutrition, pain control and antibiotics for chronic infection control such as osteomyelitis or cystic fibrosis and AIDS patients. The central veins refer to the large veins in the neck and chest in which catheters are used, *e.g.* the jugular vein, the subclavian vein, and the vena cava. There are three main modes of central venous access.

1.1 Temporary Central Venous Catheters These catheters are used for a few days up to 4 weeks and are inserted percutaneously, i.e. through the skin, over a guidewire that has been previously inserted into the vein. A sheath may be used to dilate the opening of the vein and the catheter can be inserted through this sheath. Catheters for these applications tend to be moderately stiff and are fabricated from polyethylene, polyvinylchloride or polyurethane - with a hardness greater than 95 Shore A and a modulus in the range of 0.030 to 0.100 GPa (1).

1.2 Long Term Indwelling Central Venous Catheters These catheters can be used for years and can be inserted as described for the temporary catheter, or an incision in the skin can be made and the catheter inserted directly into the vein. For most long term applications a tunnel is made from the insertion site to a site far removed, e.g. 10 cm away, and the end of the catheter is placed into the tunnel and will exit the skin at the more remote area, Figure 1. The tunnel tends to reduce the incidence of infection of the catheter in the blood site. Usually a polyester velour, e.g. Dacron®, cuff is attached on the catheter about 2 to 3 cm from the exit site of the catheter. Collagen grows into the cuff during wound healing and results in securing the catheter to the surrounding tissue in order to reduce the chance of accidently pulling the catheter out of the vein and to produce a barrier to bacterial migration from the skin exit site towards the vein . These catheters have a hardness of less than 85 Shore A and a modulus that ranges from 0.008 to 0.020 GPa (1, 2). Softer and more flexible catheters are required for long-term indwelling catheters in order to reduce damage to the blood vessels.

1.3 Implantable Vascular Access Ports These are totally implantable long term central venous access systems. The catheter portion in inserted in the same manner as above, except the catheter does not pass through the skin but instead is connected to a subcutaneous port, Figure 2. The port is a chamber made of stainless steel, titanium or polysulfone with a silicone rubber septum that can be accessed through the skin by a non-coring needle. Since needle placement is temporary and the needle tract can heal, the incidences of infection are reduced with this system. However, skin puncture is needed each time the port is accessed.

2. DESIGN ISSUES FOR CENTRAL VENOUS CATHETERS

There are a number of different properties that central venous catheters require to function effectively with minimal side effects (3, 4, 5, 6), Figure 3. Thromboresistant properties will

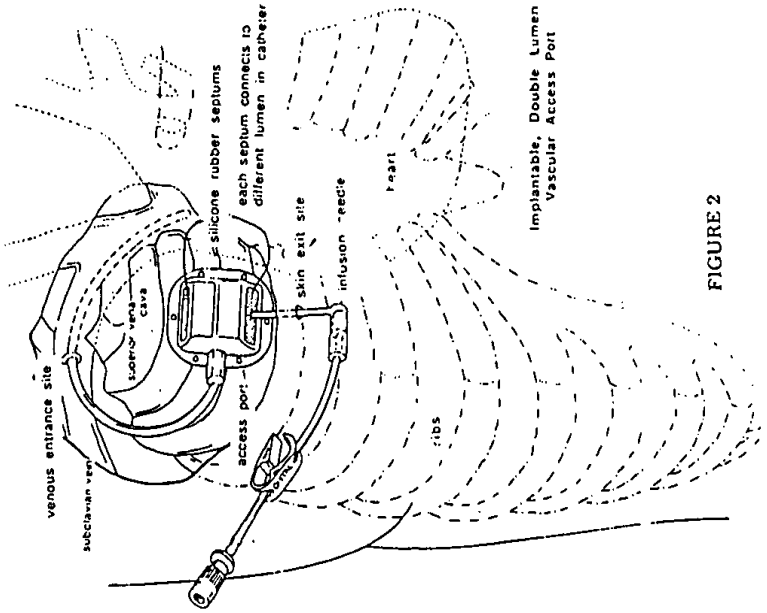

FIGURE 2

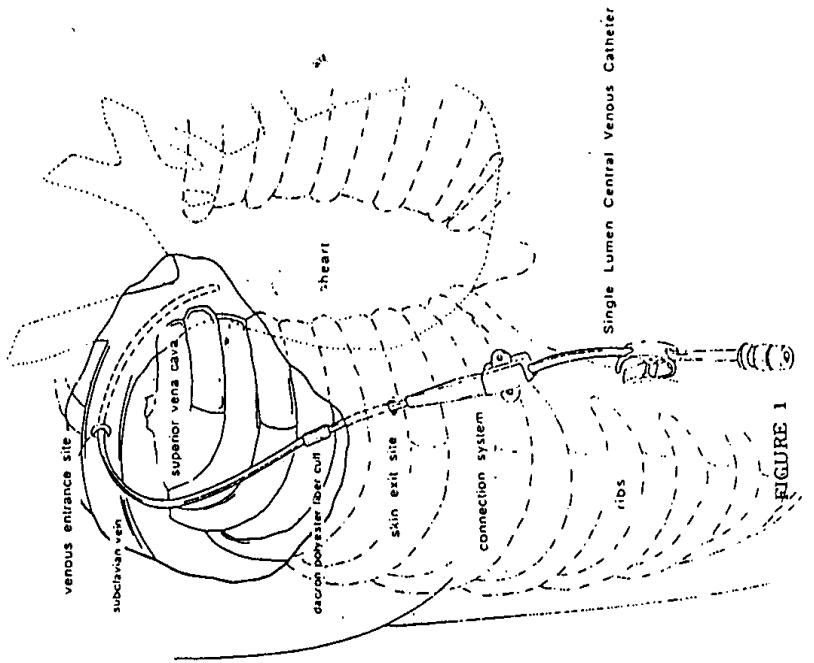

FIGURE 1

complications due to thrombus which can lead to blockage of blood vessels and emboli which can lead to organ damage and stroke. However, other material and catheter properties influence the function of these catheters.

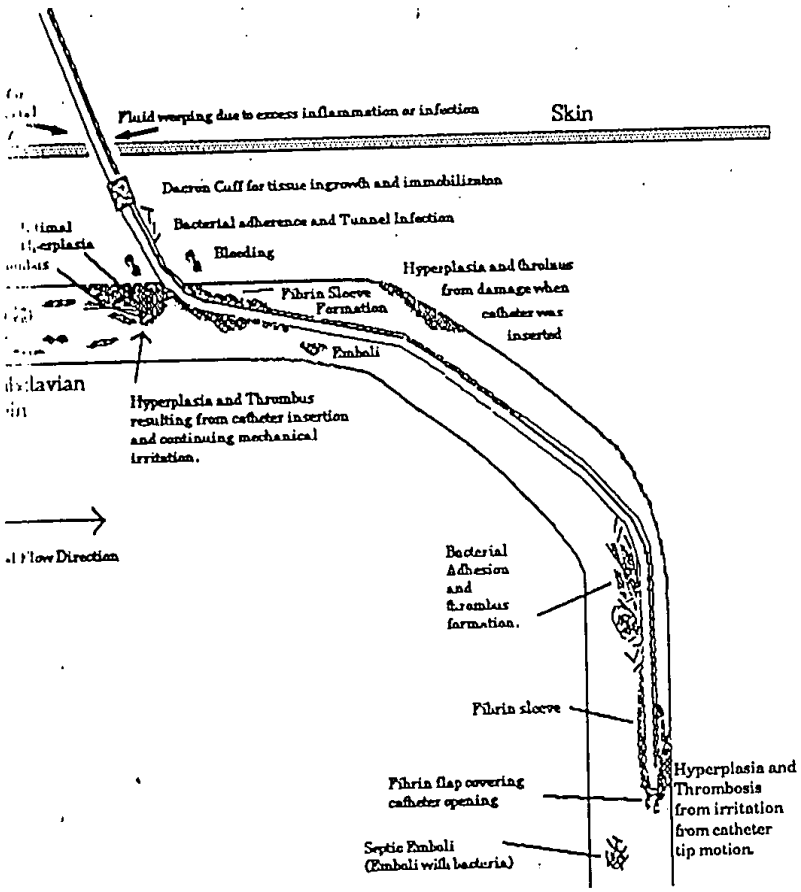

FIGURE 3 Potential complications resulting from the use of central venous catheters.

following properties can effect the ultimate success of blood contacting catheters:

insertion technique and resulting damage to the vessel.

size of catheter in relation to blood vessel and flow rate over the catheter

- more turbulent or static areas can result in thrombosis

duration of catheterization

- risk of thrombosis increases with time

mechanical properties of catheter

- longitudinal stiffness (stiff materials can pierce the vessel wall or damage the vessel wall; vessel wall damage can result in thrombosis and eventual tissue thickening known as hyperplasia)
- kink resistance
- memory of preformed curves after insertion.
- burst strength
- softening of the catheter due to water uptake and warming

- surface chemistry and effect on blood-materials interaction
- roughness, pits, and fissures which can result in a nidus of thrombus
- toxic leachables
- degradation resulting in toxic leachables and loss of mechanical properties
- lubricity
  - drag of catheter could damage tissue
- effect of manufacturing process on physical and biologic properties
- sterilization methods
  - change in mechanical properties, degradation, and toxic leachables
- patient health and medication

Other properties for a successful central venous catheter include:

- Ease of infusing fluids and withdrawing blood through the device
- Puncture resistance of port septums for the totally implantable vascular access ports
- easy repair of the externalized catheter portion for the externalized access catheters
- patient comfort and ease of care

The selection of materials for cardiovascular catheter applications will consider the range of necessary properties discussed above. Central venous catheters require materials that are relatively soft to reduce damage to the vessel wall which can result in thrombus, particularly due to the slow flow rates of the venous system. Soft polymers of polyurethanes or silicones are utilized for long-term indwelling central venous catheters. The class of polymers known as polyurethanes contain a large number of related but different polymers. In general, properly processed polyurethanes that are smooth surfaced, without filler particles at the surface, demonstrate thromboresistance equal or greater than silicones as demonstrated by platelet uptake and thrombus formation. Polyurethanes offer advantages over silicones since they can be fabricated to be relatively stiff, making for easier insertion. However, they soften upon entering the body due to body temperature and the effect of water uptake. This reduces mechanical trauma to tissue for long-term indwelling applications. However, the processing conditions can significantly alter polymer properties, as was evidenced by the degradation of polyurethane pacemaker leads.

3. KEY DESIGN ISSUES

3.1 Thromboresistance Thrombosis related complications are the most frequently encountered problems with venous catheters. Studies report various incidences, some as high as 48% and can result in serious complications to the patient (7). Thrombus can occur either in the device, in a vessel around the device or at the introduction site to the blood vessel. Occasionally, the clot will grow and propagate along the body of a catheter, surrounding it in a sheath, potentially occluding the distal outlet(s) of the device, Figure 3. As the clot ages, its composition changes, composed predominantly of collagen. Often this sheath will not impede infusion through the device, but when aspiration is attempted, the sheath will be drawn into the side or end hole openings and occlude blood withdrawal. The formation of a mural clot within the lumen of catheter can also occur. If an occlusion occurs, this can usually be remedied by the immediate infusion of clot dissolving drugs such as streptokinase or urokinase.

Several steps can be taken to minimize the risk of catheter related thrombosis: the catheter itself can, by selection of materials, geometry and fabrication processes, reduce the potential for thrombosis. The clinician, by proper match of product to patient and careful technique, can minimize the damage caused to the vessels during catheter introduction. Later, by proper maintenance of the device, *e.g.* daily flushing of the device with heparinized saline, patency of the device and the blood vessel can be maximized. Despite such precautions, thrombosis often occurs, and frequently intervention of some sort is required to restore device function. Issues which have been shown to affect thromboresistance include flow effects; surface properties; surface texture; blood vessel trauma; and infused drugs.

3.1.1 Flow effects Stagnation and turbulence are known to initiate thrombosis. Both of these conditions can occur as a result of an indwelling catheter. A catheter which is large enough to occlude flow substantially can result in adequate stagnation for clotting to occur. The geometric design of a catheter can influence blood clotting. Abrupt changes in shape can produce turbulent flow patterns resulting in high enough shear stresses to the blood components to produce damage and resulting thrombus. Also, cavities such as side holes and end holes may become filled with blood which may coagulate.

3.1.2 Surface Properties and Modifications There has been extensive research into the role of surface properties on thrombosis (6). Most central venous catheters are composed of silicone or polyurethane. Catheters contain radioopaque fillers so that they can be seen by X-Ray. However, poorly processed catheters have exposed fillers at the surface, usually barium sulfate, and these particles are particularly thrombogenic (5). When processed to have smooth defect free surfaces, some polyurethane systems have demonstrated superior thromboresistance, as related to lower fibrin and platelet aggregates on the surface, compared to silicones (8). Some newer products have incorporated hydrophilic coatings which have been described as being thromboresistant (9). Though sufactant-heparin coatings have been available for the past decade, newer catheter coatings utilizing controlled release of heparin to maintain an anticoagulant environment have been introduced. Harbor Medical Devices describes the application of drug delivery technology to improve the function of medical devices as *Controlled Environment Devices.* Experimental materials are in development and are based on a variety of properties including glow discharge coatings that are more hydrophobic than Teflon®; negatively charged polyurethanes; and permanent immobilized coatings of anticoagulants (agents that prevent clot formation, such as heparin) and thrombolytic agents (agents that dissolve clots) (6).

3.1.2.1 Heparin Release Coatings Sufactant-Heparin complexes are based on surfactants of either benzalkonium chloride (BC) or tri-dodecylmethyl ammonium chloride (TDMAC). Surfactants are detergent-like molecules which adsorb to surfaces (a molecule related to a detergent with a water "liking" and and water "hating" end). The water "hating" portion of the molecule attaches to the surface of many polymers and the water liking portion extends outward, providing the anchor to bind the heparin. These surfactants are positively charged, i.e. when they are dissolved in a salt solution, they would be attracted to the negative terminal of a battery. Conversely, heparin is a negatively charged molecule and binds to the positively charged surfactant molecules. The surfactant-heparin coatings contain a layer of surface bound heparin that releases a small amount into the blood during the first day of exposure while the remainder does not appear to release. Figure 4 demonstrates how these coatings function.

Controlled release heparin coatings contain a larger reservoir of heparin and can slowly release heparin into the blood for longer periods of time, e.g. weeks. These coating utilize controlled release technology that has normally been utilized in drug formulations. Table 1 compares the amount of heparin contained in surfactant-heparin coatings to controlled release coatings. Harbor Medical Devices has addressed the development of thromboresistant surfaces by developing a coating that releases heparin at a rate shown to reduce thrombosis, 2.4×10^{-4} mg/cm^2/h (10). The larger the reservoir the longer the time that the heparin release can be controlled. Catheters with these coatings are available from Harbor Medical Devices and Toray Industries. Figure 5 schematically demonstrates how Harbor's controlled release coatings function. The heparin coating from Harbor Medical Devices, Harborin®, utilizes an outer polyurethane membrane to meter the release of heparin contained in an underlying polyurethane binder. The release rate profile of Harborin compared to Toray Industries' Anthron® coating is shown in Figure 6. Anthron is a positively charged polymer that ionically bonds the negatively charged heparin and releases the heparin by ion exchange with the ions in blood. Release rates were determined by elution of a 4 cm length of coated catheter into 7 cm^3 of phosphate buffered saline (PBS) under sink conditions and agitated at 37° C. An Azure A colormetric assay was used to quantitate the amount of heparin released into PBS (11). Harborin released effective levels of heparin for one month.

Table 1

Available Heparin in Commercial Coatings

Coating Type	Available Heparin
	milligrams per square centimeter
Controlled release coatings	
Harborin	2.5
Anthron	0.35
Surfactant-heparin coatings	
TDMAC	0.05
BC	0.01

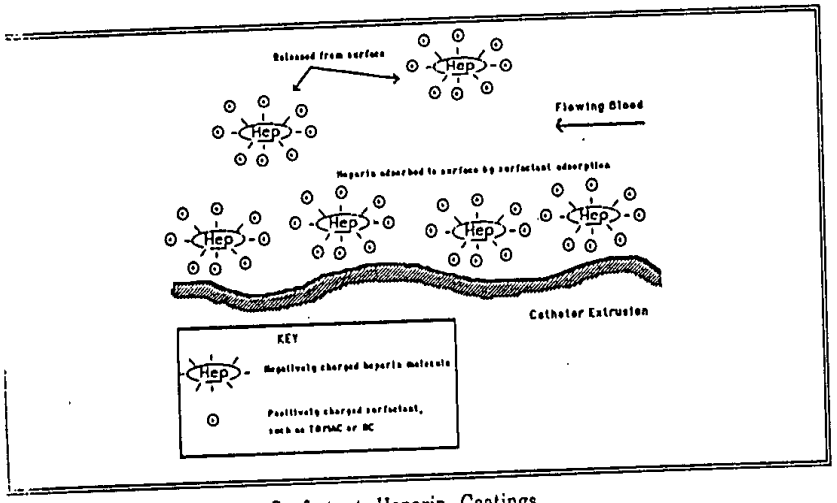

FIGURE 4 Surfactant -Heparin Coatings

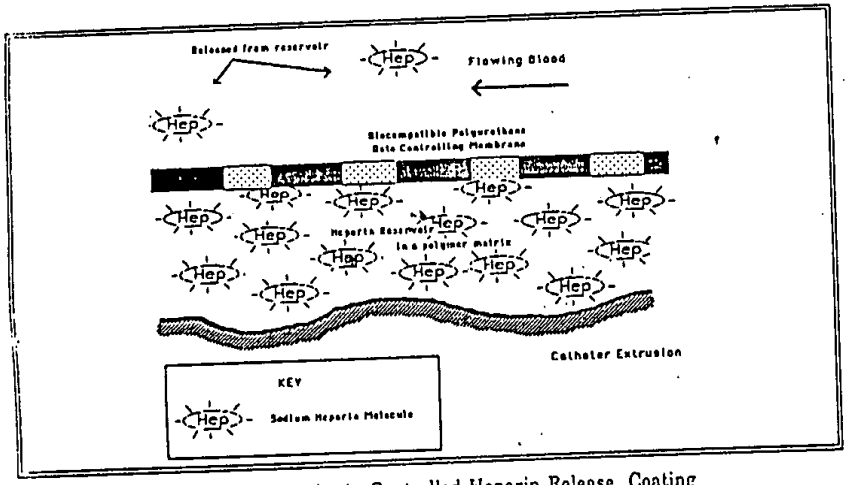

FIGURE 5 Harborin Controlled Heparin Release Coating

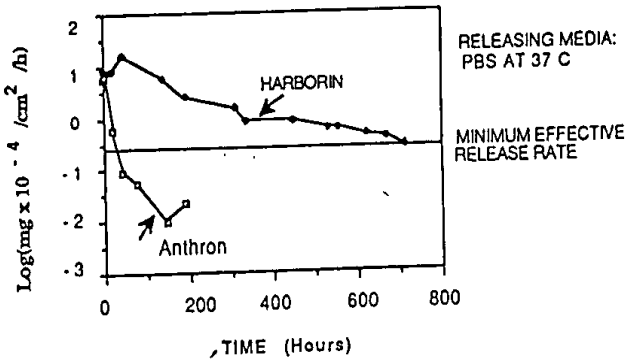

FIGURE 6 Heparin Release Profile from Harborin and Anthron Coatings.

3.1.3 Surface texture It is believed that surface roughness can form a nidus for thrombus as well as cause turbulence in the blood flow which can result in sufficient damage to blood components to produce thrombus. Surface defects on the order of less than 0.001mm can result in air bubble retention in the surface defect. The site of an air bubble on the surface can become a nidus for thrombus formation (12).

3.1.4 Blood vessel trauma Thrombus can result from injury to the native vessel wall which may be produced by the catheter or by components used during catheter introduction. Some damage to the native vessel is unavoidable as a result of the introduction process. Further damage may result from forceful manipulation to position the catheter tubing into position. A catheter tube which is too stiff may produce damage even after placement is complete if normal motion of the vessels causes a rubbing of the catheter into adjacent blood vessel tissue. This trauma may result in tissue thickening, known as intimal hyperplasia, at the site of the catheter entry into the vein and at other sites of blood vessel damage. A catheter which is too stiff, can accidently pierce the vessel during placement, with potential severe complications for the patient.

3.1.5 Toxicity of infused pharmaceuticals Drugs used either as part of the intended therapy or in attempts to insure proper device function can directly affect thrombus formation or result in damage to the blood vessel which can also activate thrombus formation. There are several antibiotics and chemotherapy agents which are irritants to blood vessel tissues. A routine part of most infusion procedures involves flushing all channels with a solution (usually heparinized) which is intended to delay thrombosis. When properly performed, this "blocking" procedure is usually effective. However, inadequate concentration or volume or frequency of these injections can compromise the effect.

3.2 Catheter Stiffness The material used in Harbor's catheter products, polyurethane, provides for improved catheter properties by softening as it warms to its *in situ* environment. While many aspects of catheter design affect its biocompatibilty, flexibility is an important parameter. A catheter which is too stiff *in situ* may cause trauma to a vessel wall as a result of spontaneous motion by the patient or as a reaction to fluid injection. During insertion, however, some degree of stiffness or "column strength" is necessary for catheter placement. Flexible materials are difficult to introduce into the vascular system even with the use of an introducer sheath. And because they seldom stay in place during introducer removal, they almost always require subsequent repositioning. Stiffer materials offer the possibility of percutaneous introduction, or a simpler procedure if used with a sheath. The polyether urethane used in the construction of Harbor's central venous catheter softens by a 50% after a few minutes exposure to an aqueous environment at 37° C. The modulus of the catheter material at ambient conditions and at 37° C in PBS was determined by application of the equation which describes the deflection of a cantilever* (1,2). For a 2.5 cm long catheter segment, 0.19 cm O.D. and 0.12 cm I.D. made from a an 80 A Shore Hardness polyurethane, the dry modulus was 0.015 GPa for a deflection of 0.5 cm. The modulus of the catheter soaked in PBS at 37° C for 15 minutes and tested in the same manner as above was 0.0076 GPa.

The mechanism of polyurethane softening relates to two distinct actions. These polyurethanes are comprised of two basic groups, or "blocks", of molecules. One of these blocks is referred to as the "soft block"with a glass transition significantly below 0° C and the other the "hard block" with a glass transition above 100° C . With increasing temperature the soft blocks actually expand, resulting in greater mobility of the material, and hence greater flexibility. Furthermore, the material absorbs a small amount of water, less than a 5 %, *in situ*. The water acts as an internal lubricant, *i.e.* a plasticizer, resulting in a further decrease in stiffness.

By designing catheters from polyurethanes, the product benefits from the well known biocompatibility of these materials. Further, because of the changes in stiffness which occur between ambient and in situ conditions, a product can be produced which suits both the mechanical requirements of introduction by supplying greater stiffness to push the product into place, and biological requirements of reducing the potential for damage from product motion by softening significantly once introduced.

3.3 Infection Resistance The need for blood access continues to grow with new diagnostic and therapeutic procedures; however, complications, particularly infection, often limit the success for chronic, *i.e.* long term, applications (13-16). Infection rates in central venous catheters are higher than those used for short duration, less than 3 days, and average about 20% in some institutions (17,18). A major contributing factor to the sepsis rate is skin site

* Meideros and Grenon (2) studied the catheter stiffness changes of Harbor Medical Devices' catheters in aqueous solution.

contamination which tracks down along the catheter to reach the vasculature (13, 19). The need to reduce infection of these devices is acute since many of the recipients are immuno-compromised due to chemotherapy or AIDS .

Dacron® polyester velour cuffs are available on some of these devices for tissue stabilization, inhibition of epidermal downgrowth, and a barrier to bacteria. They are placed on the catheter so that they remain in the subcutaneous tissue tunnel, Figures 1 and 3, and allow the surrounding tissue to grow into the velour structure. Most long-term indwelling central venous catheters are supplied with a preattached Dacron cuff. The preaatachment requires the surgeon to place the skin exit site such that the cuff will remain in the subcutaneous tunnel. The exit site may not be optimal for the patient for ease of use or comfort. Harbor Medical Devices developed a cuff with a pressure sensitive adhesive resistant to an aqueous environment that allows the surgeon to place the cuff at a location which optimizes the exit site for the patient.

Externalized medical devices, e.g. central venous catheters, which require handling show higher infection rates, which increase dramatically with the duration of use. Infection rates can increase three fold for a device used used for one month versus one used for one week and removed. The effect of handling can be seen as infection rates increase with central venous catheters with three lumens, i.e. three openings for infusion of drugs or blood withdrawal, versus catheters with only one lumen. Design improvements used to limit handling and sites of contamination, e.g. connectors, can be expected to significantly reduce infections associated with medical devices.

An approach to reducing infection is the development of devices which release antimicrobial agents (antibiotics and broad spectrum chemicals). This concept is described by devices which incorporate bioactive agents which slowly release over time in order to beneficially modify biological interactions adjacent to medical implant devices but without effect on the rest of the body, *i.e.* no systemic effect. Many of the efforts in this area entail the use or modification of antimicrobial solutions used to cleanse skin and wounds. These systems are based on broad spectrum antimicrobials. These have a broad range effect against many different bacteria as well as fungi and viruses. Vitaphore has developed an antimicrobial cuff placed on the catheter at the skin exit site. The antimicrobial action results from the biodegradation of an erodible collagen matrix and the subsequent release of antimicrobial silver ions (20).

Harbor Medical Devices has applied the concept of *Controlled environment devices* to the release of antimicrobial agents. Long term sustained release, up to two months, capable of killing bacteria adjacent to medical devices can be obtained by coating medical devices based on the system shown in Figure 7. The key to these devices is the ability to release antimicrobial agents at a rate higher than the minimum required to kill microbiologic

agents, such as *Staphylococcus epidermis*, that are virulent in the presence of a biomedical material.

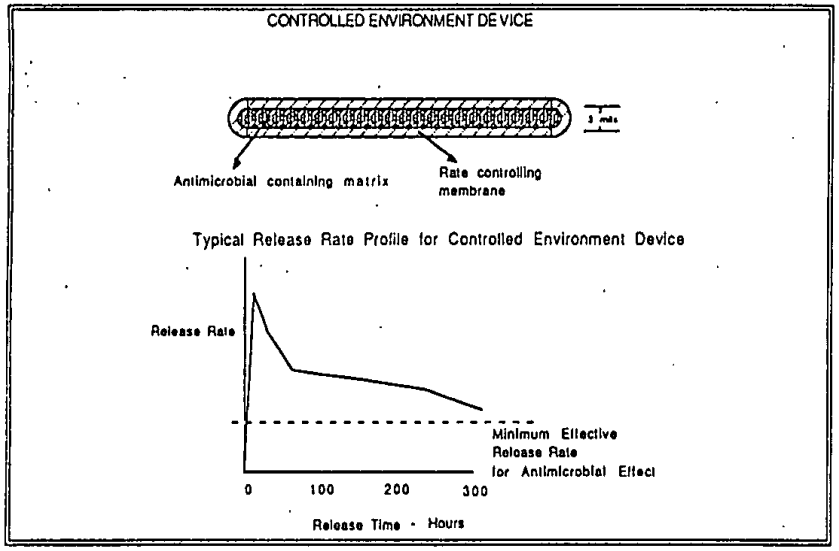

FIGURE 7 Controlled Environment Device for Release of Antimicrobials

3.4 Other Issues There are numerous properties that can effect the success of central venous catheters. Dual lumen catheters allow infusion of incompatible drugs simultaneously. The offset of the distal openings is advantageous to prevent the premature mixing of these drugs before adequate dilution in the blood can occur. Both silicone and polyurethane dual lumen catheters have these offsets. However, the use of polyurethane has other advantages than to those mentioned previously. Polyurethane is a thermoplastic that can be easily molded by thermal methods. This allows for tapering the distal tips, a process that cannot be done on silicone catheters, since these are fully cured during extrusion. Most silicone catheters do not have tapered tips. The polyurethanes also have a significantly higher tear strength than silicones and this reduces the danger of breakage due to nicking or cutting of the catheter. Tearing of the catheter could become a significant problem in implantable access ports at the site of the catheter connection to the port and the use of polyurethanes has the potential of reducing breakage at this site.

Figure 1 shows a connection system on Harbor's the externalized central venous catheter just above the skin exit site. This connection system allows for the easy replacement of the external portion if cracking or tearing occurs. The external tube above the connector is made of silicone rubber since silicone resists deformation from the clamps used to close the device when not in use, Figure 1. However, cracking and tearing of these silicone tubes occurs frequently. Most catheters do not have a connection system and the repair requires the use of room temperature curing silicone and is quite a nuisance to the physician and

nursing staff and the catheter cannot be used for a period of about 12 hours. The use of a connection system results in an easy repair and immediate use of the catheter.

There are trade-offs in the use of the external versus implantable access systems. The external catheters are easy to use but uncomfortable and prone to infection. The implantable systems are more comfortable and have a significant reduction in infection rates, but the skin must be punctured by a needle for each use. Infection is reduced since the needle is removed after each use and a relatively small puncture site heals quickly. However, if the needle dislodges from the septum during use for chemotherapy, the highly toxic chemotherapy drugs can be infused into the tissue resulting in significant tissue damage. This is rare and is unlikely to happen if the needle is secured with tape to the skin.

Most implantable access ports have the silicone septum on the upper surface. The side entry aspect of Harbor's ports, Figure 2, allows a 90° needle to rotate and lay flat against the skin. This results in significantly improved comfort for the patient. Harbor Medical Devices' port body is fabricated from titanium, a low density metal known for its tissue compatibility and resistance to corrosion and cracking.

4. CONCLUSION

Central venous catheters demonstrate the need for multiple design parameters in order to design successful devices. Technology improvements have been incorporated into new devices as demonstrated by the use of polyurethane catheters, and controlled environment devices of heparin and antimicrobial agents in order to reduce tissue damage, thrombosis and infection. There are other design factors that have been implemented that improves the placement by the surgeon and improves the use by the nursing staff and patient.

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6. BIOGRAPHY

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BIOCOMPATIBILITY TESTING OF PEKEKK

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ABSTRACT

Titanium, stainless steels, and cobalt chrome alloys are currently used for the majority of the orthopedic implants. Typical flexural moduli for these metals are 114, 193, and 232 GPa respectively, as compared to 18 GPa for a human femur. In load bearing situations, this leads to the metal implant carrying the majority of the applied loads. The local understressed bone (said to be shielded from the stresses) tends to resorb causing long-term problems. Research is being conducted on the development of polymer/carbon fiber composite implants with flexural moduli similar to that of bone in order to resolve this problem. Much of the preliminary research has focused on polysulfone/carbon fiber composites. However, we believe that the poly(phenylene ether ketone) class of polymers have much better environmental resistance than polysulfone and will therefore prove to be superior for medical implantation. The hope for good environmental resistance is based on the extreme resistance of the polyetherketones to a wide variety of solvents, and some preliminary biocompatibility testing.

KEYWORDS: Composites; Testing/Evaluation/Characterization; Polymers, Polyetheretherketones; Biomaterials

1. INTRODUCTION

One problems of increasing concern in the area of artificial hips is stress shielding. This is the reduction in stresses seen in bone immediately adjacent to stiff metallic implants that causes resorption and weakening of the bone. This remodeling is in response to the physiological Wolff's Law which says that bone and other tissues will remodel to respond to the applied stress (paraphrased as "use it or lose it").

The stress shielding is caused by the stiff metal implant carrying the load normally carried by the bone. Titanium, stainless steels, and cobalt chrome alloys are currently used

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BACKGROUND

The use of heparin bonded to various surfaces was first described by Gott using a graphite-benzalkonium chloride-heparin (GBH) [1]. Gott showed the clinical value of using vascular shunts with wall bonded heparin in six patients undergoing descending thoracic aneurysmectomy [2]. The principal disadvantages of the GBH coatings are the lack of transparency and tendency to flake when used with flexible polymers. A modification of the coating which overcomes both these disadvantages was developed by Battelle Laboratories [3,4] and is available from Polysciences, Inc. This modification consists of a transparent coating of tridodecylmethylammonium heparinate (T-H). The clinical success of these vascular shunts has been reported by Gott [5] and Krause [6]. Dr. Gott and coworkers believe that T-H coatings greatly simplify the procedure for resection of aneurysms because the need for a pump bypass system and systemic heparinization which often lead to operative and postoperative bleeding is eliminated.

The mechanism by which T-H adheres to a surface may be due to the large number of van der Waals forces produced by the lipophilic hydrocarbon chains of the tridodecylmethylammonium group.

There are a number of other techniques [7-10] described in the literature for impregnating a surface with a quaternary ammonium salt of heparin. These methods are generally more cumbersome and less effective. The solvent of choice for T-H coatings is toluene-petroleum ether 50:50 (v/v) and the b.p. of the petroleum ether is 70-96°C. More polar solvents may be used although T-H is insoluble in water, methanol and ethanol. The T-H solution supplied is not sterile and the solution should be filtered before use through a polypropylene 0.22 to 10 μ filter. Cellulose filters are not recommended.

DIRECTIONS FOR USE

Several publications describe the use of T-H [11-19]. At the 1976 American Society for Artificial Organs meeting, Roohk [20] showed that TDMAC-heparin treatment reduced the fibrinogen absorption which may be highly significant since absorbed fibrinogen may be responsible for thrombogenesis.

The T-H solution supplied can be used to coat surfaces made of silicone [21], mylar, dacron, polycarbonate [21], by simple immersion for a few minutes. To coat surfaces made of polyethylene and polypropylene, longer periods and elevated impregnation temperatures (40-50°C) are often helpful. A teflon surface can be coated by refluxing in the T-H solution for 2-3 hours. The choice of solution concentration (2, 5, or 7% w/w), in the 1:1, v/v toluene-petroleum ether solution will depend on the desired thickness of the coating. For tubing or catheters with a small lumen it is advisable to use 2% T-H to avoid further restricting the inside diameter. For non-enclosed coated surfaces, 1-3 hours of air drying should be enough. Enclosed areas can be dried by blowing a dry stream of filtered nitrogen through the enclosed area for 5-20 minutes. After drying, the device can be sterilized by autoclaving if the substrate will take the treatment or by ethylene oxide gas sterilization. If ethylene oxide is used, outgassing procedures are advised.

CLINICAL INVESTIGATION REQUIREMENTS

Dr. Gott has obtained the permission of the Johns Hopkins Hospital Clinical Investigation Committees to use TDMAC-heparin shunts for thoracic aortic and innominate artery bypass in patients. The FDA has not yet decided if this treatment falls under the device or drug laws. Until the treatment is classified as a "drug", the surgeon and his hospital committee have the responsibility for its use. All investigators are advised to seek similar approval of their Clinical Investigation Committee for use of these coatings.

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fractions were pooled, dialyzed against Type I water, and lyophilized. The copolymers were characterized by proton NMR spectra, heparin content, and biological activity, as detailed below.

Determination of heparin content of copolymers

The amount of heparin fragment contained in a copolymer was determined by the modified carbazole-uronic acid reaction[31] in the presence of sodium tetraborate (25 mM) in concentrated sulfuric acid. The assay procedure involved adding 50 μL of carbazole reagent in ethanol (1.25 mg/mL) to an ice-cooled mixture containing 1.5 mL of borate/sulfuric acid reagent and 250 μL solutions of appropriate heparin fragments (controls), copolymers with the heparin fragments, and glucuronolactone standards (0–20 μg). The mixtures were vortexed and heated for 15 min in a boiling water bath. The absorbance at 525 nm was measured at room temperature. The uronic acid content of the heparin fragments and that of the copolymers was determined by comparison with a glucuronolactone standard curve. The heparin content of the copolymers was calculated from the uronic acid content of the respective heparin fragments.

Determination of bioactivity of copolymers

Biological activity of copolymers and the corresponding heparin fragments were determined in terms of anti-Factor Xa activity and activated partial thromboplastin time (APTT), per Sigma Technical Bulletin No. 870 and Procedure No. A7668, respectively. Briefly, a solution of the test material was incubated with plasma to neutralize or inactivate the coagulation factor(s). The observed clotting time, being proportional to the amount of heparin present, is a measure of the respective activity. This was read directly from appropriate standard curves obtained by using a stock solution of commercial heparin (Sigma) of known specific activity (10 USP Units/mL). The ratio of anti-Xa activity to APTT, which relates to a substance's local antithrombotic potential with respect to its global anticoagulant or antithrombogenic activity, is calculated from these results.

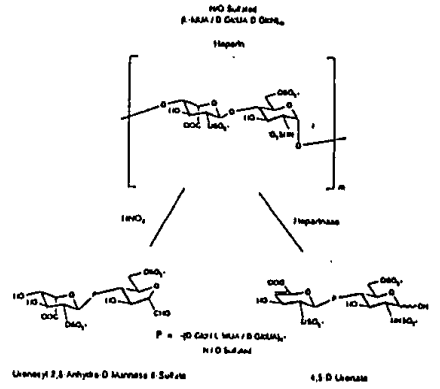
For the APTT assay, solutions of copolymers or heparin fragments in normal human plasma (25 μg/mL; 100 μL) were incubated with APTT reagent (100 μL; solution containing brain cephalin in ellagic acid buffer) for 3 min in a Fibrometer cup to which was added a solution of calcium chloride (100 μL; 25 mM). Similarly, 100 μL of activated Factor-Xa reagent was incubated for 90 s with 100 μL solution of copolymers or heparin fragments (8.3–500 μg/mL) diluted in 100 μL of normal human plasma and 700 μL of Triznal buffer (Sigma). An aliquot of the mixture (100 μL) was transferred to a Fibrometer cup, followed by the addition of 100 μL calcium chloride solution and 200 μL of plasma-CEF (rabbit brain cephalin) reagent, all solutions and mixtures being prewarmed and all incubations for both assays done at

37°C. It is to be noted that different amounts of copolymer have been used for the APTT and anti-Factor Xa activity measurements, due to the difference in sensitivity of the two assays. Also, since different amounts of copolymer or heparin fragment were used for these assays, the bioactivity has been calculated to the unit weight of the fragment or the copolymer, that is, expressed in terms of specific activity, irrespective of heparin content.

RESULTS AND DISCUSSION

Enzymatic degradation of heparin with heparinase from *F. heparinum*[23,24] and chemical depolymerization by deaminative cleavage with nitrous acid[25,26] are well described in the literature. These are shown schematically in Figure 1.

There is now a general consensus that a necessary structural prerequisite for significant anticoagulant activity is a specific pentasaccharide sequence of heparin with essential features that constitute the "minimal" binding site for antithrombin.[27,28] This specific heparin segment is sufficient for the antithrombin-mediated inhibition of Factor Xa but inadequate for the effective inhibition of thrombin and for the development of full anticoagulant activity. The latter requires a segment of at least four (trisulfated) disaccharide units in addition to the pentasaccharide sequence of the active site for antithrombin.[29,30] However, the sizes of the heparin fragments used in this study are far from those of conventional heparin (average 12,000–19,000 Da) which



(A) (B)

Figure 1. Degradation of heparin and typical products from (A) nitrous acid cleavage and (B) heparinase digestion.

Figure 1. Comparison of the different diffusion layer. The thickness of the tie coat (37.5% hep) is about 40 μm. Note: the intial release rate is ~350 μg/cm²/hr for pure tie coat (not shown)

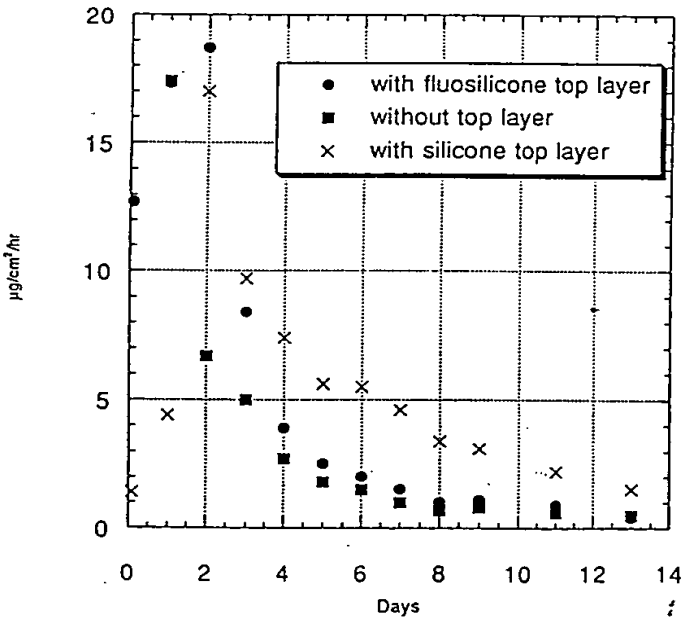
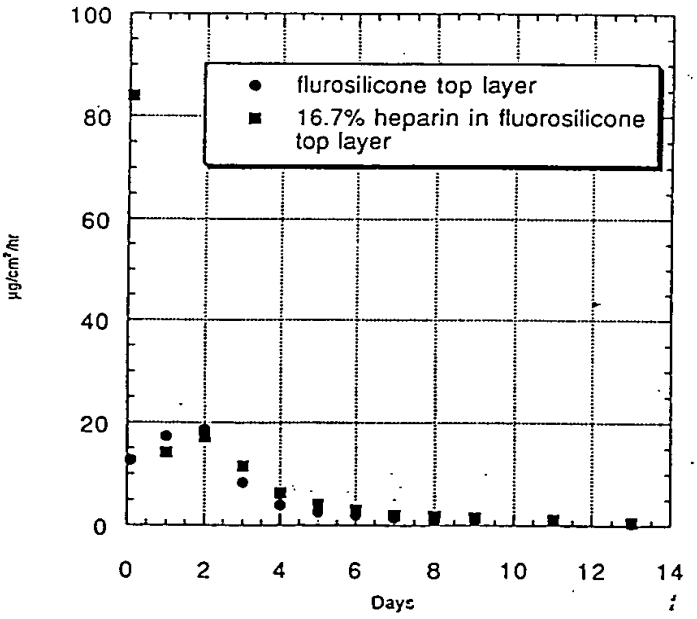


Figure 2. Comparison of fluorosilicone top layers with and without heparin. The thickness of the tie coat (37.5% heparin) is about 40μm.



The main advantage of the surface modification of the eluting heparin coating is to maintain the surface thromboresistent or non-thrombogenic during or after the biological active species elute. Several approaches can be taken to achieve it.

a.) Heparin-coated stents with or without the diffusion top layer can be covalently bound with heparin or surfactant, which can bind heparin ionically on the outer surface, using various approaches for those skilled in the art;

b.) negatively charged outer surface can be created by sulfonating or sulfating the surface to form sulfonic or sulfate groups or by oxidizing the surface to form carboxylate groups and hydroxyl groups;

c) surfaces containing high electronegativity groups can be created by fluorinating the surface;

d) immobilize the coating surface with polyethylene glycol, a protein deposition inhibitor, or phospholipid (thromboresistent). These can be done by those who are skilled in the art.

Above approaches can also be achieved by top-coating a thin layer of polymers containing said bioactive species or groups in a, b, c, and d onto drug coated stents (see example 1 and 2).

Another advantage of the proposed surface modification is to tailor the release profile of the active species. A negative charged surface may slow down the release rate of imbedded heparin due to charge-charge interaction, hence control the release kinetics.

One other way to tailor the release rate is to coat a layer of surfactant bound heparin as a temporary blocker. Examples are TDMAC heparin or benzalkonium heparin. The mechanism by which surfactant-heparin adheres to a surface may be due to the large number of van der Waals forces produced by the lipophilic hydrocarbon chains of the tridodecylmethylammonium or benzalkonium group. Since the adherence is not permanent and will be gone in hours, such approach will delay the drug release and will not increase the final coating thickness, which is important in some stent applications, e.g. coronary stent application.

Examples of the surface modification of eluting heparin coating: coat the tie-lay of the stent in a way described in our previous patents and cure the coating. The heparin content are 37.5% and the coating thickness is about 40μm. Prepare fluorosilicone spray solution (Applied Silicone #40032) by weighing fluorosilicone suspension and add tetrahydrofuran by using the equation of V_{THF} = 1.2* weight of fluorosilicone suspension. Stir the solution very well and spray-coat the stent in a way similar to the tie-coat process and cure the coated stents at 90°C for 16 hours. The coated stents are Ar plasma treated prior to gamma sterilization. Figure 1. is a plot of heparin release kinetics in PBS buffer with fluorosilicone or silicone top coat or without any top coat. The thickness of the top coat is about 10-15μm. Compared with the coating without the top diffusion barrier, coatings with both kinds of top layers showed depressed initial release rates and enhanced eluting rates for the rest of the first week . Figure 2 is a plot of fluosilicone top coat containing 16.7% imbedded heparin. As a comparison, also included in the graph is a coating with fluorosilicone only. The tie coat in figure 2 are the same as in figure 1.

Coating of commercially available materials with a new heparinizable material

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Different commercial materials, such as polyurethane (PU), plasticized PVC (PVC), glass, Gore-tex, and Dacron, were coated with a well-characterized biomaterial (PUPA) based on polyurethane and poly(amido-amine) components. Two different classes of coating were obtained due to the different characteristics of the substrates. In the case of PVC and polyurethane which are soluble in the solvent of the PUPA-coating solution, there was penetration and blending of the coating and underlying materials. In the case of glass, Gore-tex, and Dacron, which are insoluble in the solvent of the coating solution, only a superficial layer of PUPA could be obtained. The coating stability was investigated and the interaction between coating and underlying material studied by FT-IR. All the stable coatings showed the ability to bind as much heparin as PUPA material by itself.

INTRODUCTION

The challenge of biomaterial research lies in fabricating materials that maintain mechanical integrity and biocompatibility in contact with blood. The utility of these devices is complicated by thrombosis. It is generally accepted that thrombotic events are stimulated by and occur directly at the surface of the material through the initiation of the intrinsic clotting cascade and the adhesion/aggregation of activated platelets.[1] The nature of thrombotic events demands that these two synergistic factors be controlled if the biocompatibility of the surface is to be optimized.

Current research is concerned with investigating surface modifications to improve the "performance" of these devices. Of the different approaches for optimizing surface biocompatibility, surface heparinization is a well accepted strategy. Heparinized materials appear to be highly compatible with both platelets and the plasma coagulation system.[2]

Our starting point in fabricating materials with improved biocompatibility is the use of poly(amido-amine)s which have been shown in previous studies to have the capacity to form a stable complex with heparin.[3] Poly(amido-amine)s maintain their heparin-binding ability even if present as a component in a given material. N_2LL poly(amido-amine), obtained by polyaddition

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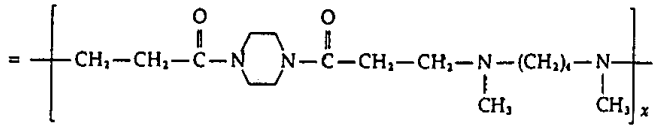
of *N,N'*-dimethyl-1,4-diamino butane to 1,4-*bis*(acryloyl)piperazine, N_2LL



was recently cross-linked with commercial polyurethane (PU) via hexamethylenediisocyanate (HMDI). The properties of the material (PUPA), obtained as a film by evaporating the solution containing the three components in a given ratio in *N,N'*-dimethylformamide (DMF), which is capable of strongly adsorbing high quantities of heparin, have already been discussed.[4] Instead of preparing new materials, the PUPA solution may be used to coat commercially available materials.

The aim of the present study was to provide a blood-compatible, heparinizable coating to improve the surface properties of existing polymer substrates without changing the bulk properties. This paper presents the initial results of preparation and surface characterization of different commercial materials [plasticized polyvinylchloride (PVC), polyurethane (PU), glass, polytetrafluoroethylene (Gore-tex), and polyethyleneterephthalate (Dacron)] coated with PUPA and their heparin-adsorbing properties.

MATERIALS AND METHODS

Materials

Pellets of the commercial polyurethane Pellethane R2363-80AE were purchased from Upjohn Polymers. The pellets were purified by extraction in Soxhlet with CH_3OCH_3/CH_3OH (v/v; 1/2.5) over a period of 3 days.

Tubes of medical-grade plasticized PVC were purchased from Baxter.

Dacron in the form of medical polyethyleneterephthalate patch fabrics was purchased from Sorin Biomedica S.p.A.

Gore-tex in the form of polytetrafluoroethylene membranes supported on polypropylene fabric (0.200 μm thick) was purchased from W. L. Gore & Ass. S.r.L.

Glass in the form of Deckglasser coverslips was purchased from Perfect.

Films of polyurethane

These were obtained by evaporation from a 10% (v/v) solution of the polyurethane pellets in *N,N'*-dimethylformamide (DMF) at 70°C *in vacuo* (400 mbar).

Films of PVC

These were obtained by slow evaporation from a 10% (v/v) solution of the medical-grade plasticized PVC tubes in tetrahydrofuran (THF) at room temperature. The film obtained showed the same IR spectrum as commercial PVC.

PUPA solution

The synthesis of PUPA involves two reaction steps, as previously described[4]:

(1) Poly(amido-amine) with terminal free isocyanate groups were obtained by adding a large excess of hexamethylenediisocyanate to the dry and alcohol free methylene chloride solution of the amino end-capped poly(amido-amine) N_2LL under nitrogen.

(2) 10% (v/v) of Pellethane® 2363-80AE in *N,N'*-dimethylformamide (DMF) was added to the above methylene chloride solution under nitrogen and vigorous stirring. The mixture was kept at 60°C under nitrogen and vigorous stirring for 3 h. After this time it could be used to coat the different substrates.

Coating procedure

(1) Polyurethane films, PVC films and glass plates were coated by pouring the PUPA solution in DMF at 60°C over the different materials under a nitrogen stream, in a sufficient quantity to cover the entire substrate surface. Evaporation of the solvent (DMF) was performed at 60°C *in vacuo* (400 mbar).

(2) Dacron and Gore-tex fabrics were bathed in the PUPA solution at 60°C under magnetic stirring for 5 h (without any further treatment) and then rinsed with DMF. Both these steps were carried out under a nitrogen stream. The DMF was then evaporated off at 60°C *in vacuo* (400 mbar). PUPA solutions in DMF at three different concentrations (80%, 50%, and 30% PUPA by volume) were used to coat the Dacron fabrics.

Slices 40 or 50 μm thick of the PUPA-coated PU and PVC samples were obtained by a microtome (Sartorius).

Extraction

(1) Samples of PUPA-coated polyurethane, Dacron, and Gore-tex were extracted in Soxhlet with CH_3OH/H_2O (6:1; v/v) for 5 h.

(2) Samples of PUPA-coated PVC were extracted in Soxhlet with pure water for 5 h.

(3) Samples of PUPA-coated glass were simply washed with CH_2Cl_2.

Stability of PUPA coating

Films of PU and PVC and Dacron and Gore-tex fabrics coated with PUPA were flexed and elongated.

Cracks did not appear for PU, PVC films, and Dacron fabrics. They only appeared for Gore-tex if it was strongly flexed. The stability of the layer of PUPA on Dacron and Gore-tex fabrics was investigated as follows: the samples, once weighed, were placed in a 7-mm-i.d. Silastic (tetramethylsiloxane) tubing circuit connected with a saline solution reservoir, and the apparatus was thermostatted at 37°C. A peristaltic pump (rate = 25 rpm; flow = 100 mL/min) was used to perfuse the circuit with saline solution. The experiments were stopped after 1 month. The samples were then removed, rinsed with 0.01 M NaOH solution, washed with water, dried *in vacuo* (400 mbar), and weighed.

The layer of PUPA was removed from glass plates by scratching when the sample was put in contact with water, CH_2Cl_2, and DMF.

SURFACE CHARACTERIZATION

Optical microscopy

Surfaces of PUPA-coated and native Dacron samples were analyzed by inverted optical microscope (Nikon: Diascopic Die Nomarski attachment TMD-NT). Photomicrographs were taken of representative areas of the surface.

FT-IR/ATR spectroscopy

ATR spectra of the samples were recorded on a Perkin-Elmer FT-IR M 1800 between 4000 and 750 cm^{-1}. An MCT detector was used and the apparatus was purged with nitrogen. Typically 300 scans at a resolution of 2.0 cm^{-1} were averaged and the spectra were stored on a magnetic disc. The frequency scale was internally calibrated with a reference helium-neon laser to an accuracy of 0.01 cm^{-1}. A KRS-5 crystal was used at an angle of 45°.

Spectral subtraction

When taking the spectrum of a coated material, the beam sees the coating layer and the underlying material for the penetration depth of the beam into the sample. It is therefore necessary to subtract the spectrum of the underlying material in order to analyze any changes in the spectrum of the coating, or vice versa.

The spectra of the native and coated substrate were taken at first. Subtraction was then carried out according to the null criterion, by suppressing appropriate bands to baseline level. In order to remove the characteristic bands of the substrate, a particular band near bands of interest is selected and nulled. This should lead to the removal of the other substrate bands, however, some negative and/or additional positive peaks of the substrate may appear in the difference spectrum due to the spectral differences between the spectrum of the native substrate and that of the substrate which interacts with the coating material.

Titration of the aminic nitrogen atoms

The determination of the poly(amido-amine) basic nitrogens present on the surface of the coated-materials was performed by dipping samples of known surface in 10 mL of 1 M HCl for 1 h. Then the samples were washed with water and dried *in vacuo* (12 h).

The samples were then treated with 0.1 M NaOH for 1 h. Chloride ions in this solution were finally determined by titration with 5×10^{-3} M mercury nitrate solution previously standardized[9] against KCl.

This titration method is capable to determine only the quantity of nitrogens present on the sample surface. A determination of the basic nitrogens in the deeper layers by a deeper penetration of the titrant[10] was not considered useful here because the different physicochemical characteristics (i.e., swelling, porosity etc.) of the materials blended to PUPA can mislead the titration results.

Heparinization and heparin determination

The heparinization of PUPA-coated polyurethane, PVC, Dacron, and Gore-tex was obtained by dipping the samples in a 0.5% solution of heparin (sodium salt, from Roche containing 5000 units = 50 mg/mL) in H_2O/C_2H_5OH (1:1; v/v) containing 2% of CH_3COOH at 60°C.

Samples of PUPA-coated PVC were also heparinized using the above experimental conditions except with a 0.5% heparin solution in H_2O containing CH_3COOH in the same amount of which above.

The amount of heparin adsorbed on the surface of the PUPA-coated materials was biologically tested by thrombin time (TT) measurements. The samples were stirred in phosphate buffered saline (PBS) solution, to remove all the heparin not bound to the surface. The solution was changed every 60 min until no heparin was monitored by biological test (see below). Secondly the samples were treated with 10 mL of 0.1 M NaOH solution changing it each hour. The solutions were neutralized with HCl solution, and phosphate buffer (pH = 7.4) was added to the solution before determining

the quantity of heparin. The treatment with 0.1 M NaOH solution was repeated till no heparin was monitored by TT measurements.

Thrombin time measurements

The TT test with "Thrombin Reagent" (from Boehringer Mannheim) was performed with an Automatic Elvi 820 Digiclot 2 Coagulimeter (from Logos S.p.A.) A standard curve, obtained by plotting dilutions of heparin (units/mL) versus time, was used to determine the heparin in the eluates.

Obviously, this is a way to determine only the heparin that is still biologically active after the adsorption/desorption process.

RESULTS AND DISCUSSION

By pouring PUPA solution in *N,N'*-dimethylformamide (DMF) on to the surface of the different materials (plasticized polyvinylchloride (PVC), polyurethane (PU), glass, Gore-tex, and Dacron), two different classes of coating were obtained. Due to the solubility of PVC and PU in DMF, the PUPA solution not only covers these surfaces, but also penetrates into the bulk, creating a blend of the two materials. In the case of glass, Gore-tex, and Dacron which are insoluble in DMF, only a layer of PUPA on the surface can be obtained.

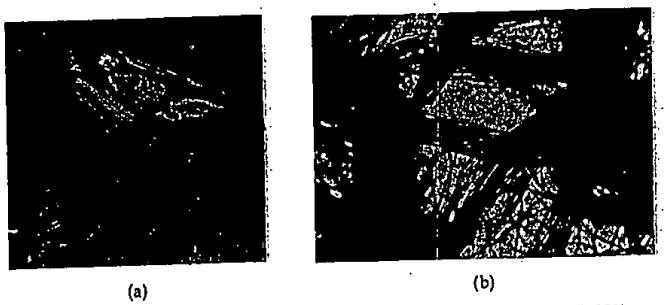
The coating of Dacron with PUPA solution first generated a more rigid material due to the thick bridges of PUPA between the Dacron fibers. Hence, PUPA solutions in DMF at different concentrations were used to coat Dacron samples in order to find the right concentration for binding large quantities of heparin without destroying the good mechanical properties of the substrate. Figure 1 shows optical micrographs of Dacron samples coated with 80% (a) and 30% (b) PUPA solutions. The thick bridges between the fibers are not evident in the sample coated with the 30% solution: a thin homogeneous layer of PUPA envelops each Dacron fiber (Fig. 1b), conserving the elasticity of the Dacron material.

FT-IR/ATR spectroscopy

Figure 2 shows the ATR/FT-IR spectra of native and coated PVC, PU, glass, Gore-tex, and Dacron, along with the spectrum of PUPA itself. All the coated-materials spectra reveal the presence of PUPA on the material surface.

Difference spectra and interaction between coating and substrate

PVC and PU. The depth of penetration of PUPA into PVC and PU samples was revealed by slicing the PUPA-coated materials with a microtome and analyzing the layers of known thickness (40 and 50 μm) by FT-IR. Figure 3



(a) (b)

Figure 1. Optical micrographs of Dacron samples coated with: (a) 80% solution of PUPA in DMF, (b) 30% solution of PUPA in DMF.

shows the spectra of these layers for coated PVC and PU samples. In both coated materials, the top layer shows a spectrum very close to that of the native PUPA. The intensity of the PUPA bands decreases, while the intensity of the PVC or PU bands increases with increasing depth, and finally, the layer at about 350 μm shows a spectrum identical to that of the corresponding substrate. This is in accordance with the continuous change in chemical composition of the coated substrate from its surface to its bulk, due to the penetration of PUPA.

(*i*) *PUPA-coated PVC*: The variations in the PVC spectrum upon coating were analyzed by taking the difference spectrum between PUPA and the coated sample. The difference spectrum is reported in Figure 4 together with that of native plasticized PVC. Comparison of the two spectra shows that the intensity of the plasticizer [dioctylphthalate (DOP)][6] bands are higher in the spectrum (a) than in the spectrum (b).

In ATR, the depth of penetration for a nonabsorbing medium, defined as the distance required for the electric field amplitude to fall to e^{-1} of its value at the surface, is given by Harrick[7]:

$$d_p = \frac{\lambda/n_1}{2\pi[\sin^2\theta - (n_1/n_2)^2]^{1/2}}$$

where n_1 is the refractive index of the sample and n_2 is the refractive index of the ATR element. It has been shown that the sampling depth for polymeric materials is about three times d_p.[8] If we were analyzing polymeric materials with refractive indices of approximately 1.5, the sampling depth of bands at 1720, 1260, and 960 cm^{-1} would be 3.46, 4.64, and 6.09 μm for a sample analyzed on a 45° KRS-5 element. Considering the penetration of the IR beam, according to this approximation, we can say that the layer investigated was close to the surface. In this layer the different intensity of corresponding peaks suggests an enrichment of DOP within the near-surface sampling depth.

This could mean that the DMF solvent of PUPA preferentially extracted the plasticizer which migrated to the surface interacting with the PUPA-coating material. As a matter of fact, in the ester C=O spectral range of the dif-

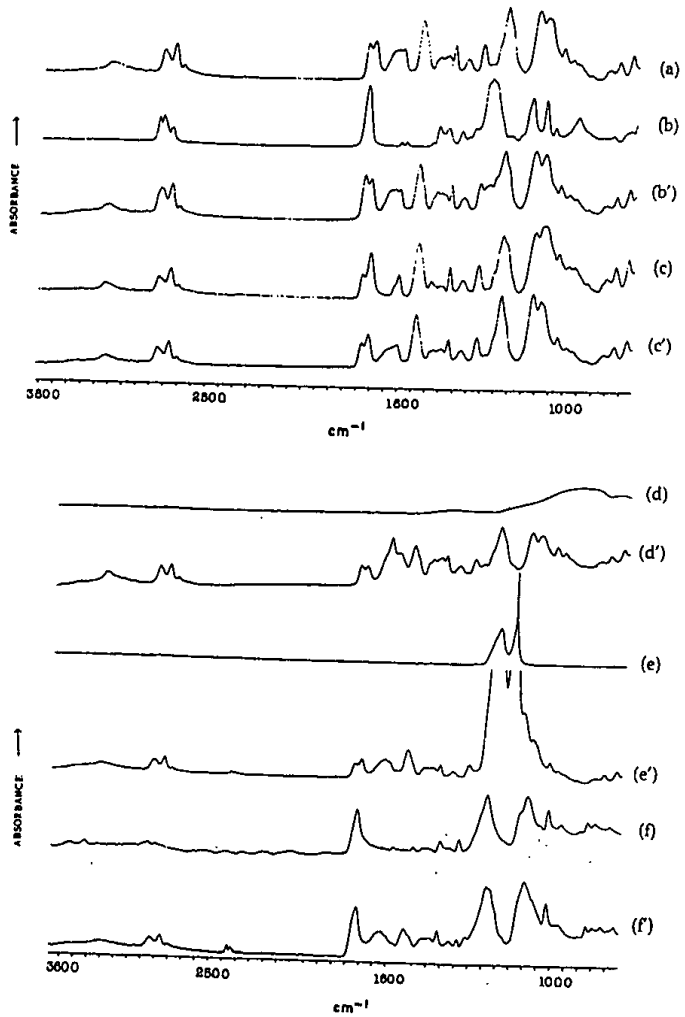


Figure 2. FT-IR/ATR spectra of: (a) PUPA, (b) PVC, (b') PUPA-coated PVC, (c) polyurethane, (c') PUPA-coated polyurethane, (d) glass, (d') PUPA-coated glass, (e) Gore-tex, (e') PUPA-coated Gore-tex, (f) Dacron, (f') PUPA-coated Dacron.

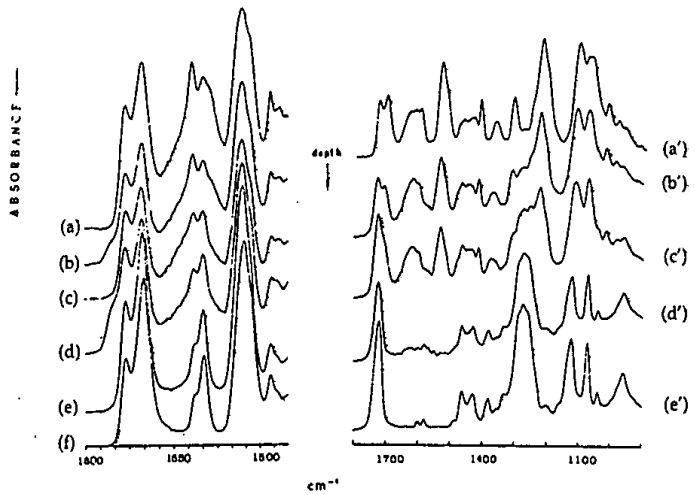


Figure 3. FT-IR/ATR spectra of PUPA-coated polyurethane and PVC. (a) PUPA, (b) top layer, (c) layer 180 μm deep, (d) layer 280 μm deep, (e) layer 380 μm deep of PUPA-coated polyurethane, (f) polyurethane, (a') PUPA, (b') top layer, (c') layer 250 μm deep, (d') layer 350 μm deep of PUPA-coated PVC, (e') PVC.

ference spectrum a shoulder appears at 1706 cm^{-1}; this shoulder is absent in the spectrum of the native plasticized PVC and can be attributed to a hydrogen-bonding interaction between the ester C=O group of DOP and the urethane and/or urea NH group of PUPA. However, this does not mean that separation of the plasticizer from the polyvinylchloride occurred upon coating, but that the coating process only provoked a partial migration of the plasticizer to the surface. The spectrum of the 350-μm deep layer of the coated sample (see Fig. 3) still shows in fact the characteristic bands of plasticizer together with those of polyvinylchloride.

(*ii*) *PUPA-coated PU:* Since PU is a component of PUPA, in the case of PUPA-coated PU the interactions between coating and underlying material occurred via polyurethane–polyurethane hydrogen bonds.[9] Subtraction of the substrate spectrum from that of the coated sample also gave information about interaction between the cross-linked poly(amido-amine) and polyurethane chains.[10]

The difference spectrum of poly(amido-amine) (Fig. 5a) shows the amide C=O stretching at 1623 cm^{-1} whereas this band falls at 1634 cm^{-1} in the free polymer[10] (Fig. 5b). The shift to lower frequency of this band can be attributed to the formation of a hydrogen bond between the amide C=O group of poly(amido-amine) and the urethane N—H group of polyurethane. As a matter of fact, the difference spectrum also shows a band at 1725 cm^{-1},

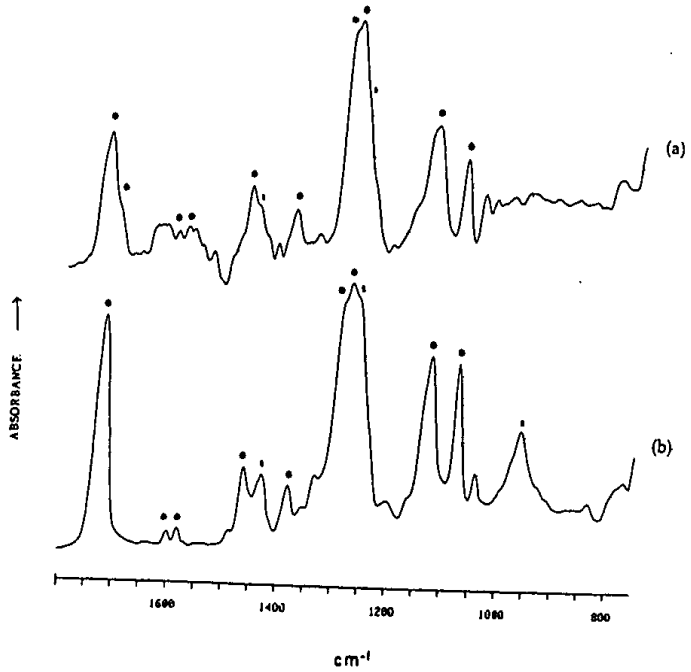


Figure 4. (a) Difference spectrum of PVC obtained from the spectra of PUPA-coated PVC and PUPA, (b) spectrum of native plasticized PVC. * bands due to the DOP, ■ bands due to the PVC.

due to the C=O stretching of the free urethane group.[10,11] The presence of this band is consistent with the breaking of some urethane–urethane hydrogen bonding in favour of the above urethane–amide interaction.

Glass, Gore-tex, and Dacron

In the case of PUPA-coated glass, Gore-tex, and Dacron, the spectrum of the PUPA coating was obtained by subtracting the spectrum of the substrate from that of the coated sample. Figure 6 shows the difference spectra of PUPA coating on glass (PUPAg), Gore-tex (PUPAG), and Dacron (PUPAD).

In the case of PUPA-coated glass and Dacron, the difference spectrum of PUPA coating does not reveal any particular variation with respect to the spectrum of native PUPA and the difference spectra of glass and Dacron obtained by subtracting the spectrum of PUPA from that of the coated sample were also equivalent to those of the corresponding native substrates. Hence we may assume that only a physical interaction occur between the coating

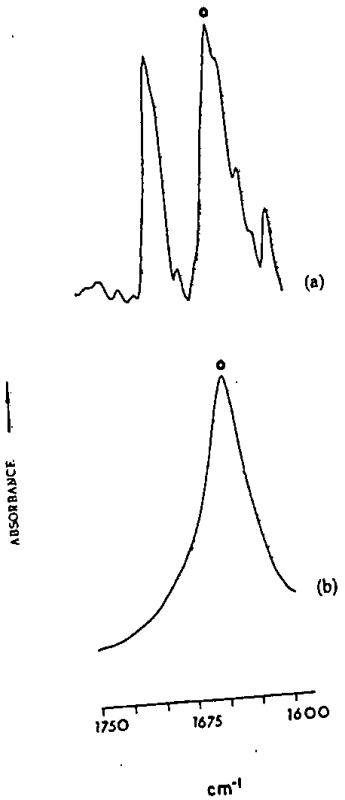


Figure 5. C=O spectral region of: (a) difference spectrum of poly(amido-amine), (b) spectrum of poly(amido-amine) by itself. O band due to the amide C=O stretching.

and the substrate or that the chemical interactions if any, are too weak to be detectable by FT-IR.

In the case of PUPA-coated Gore-tex, however, the difference spectra of PUPA coating and Gore-tex substrate are not the same as those of native PUPA and Gore-tex. In the difference spectrum of PUPA coating (PUPAG) (Fig. 6b), some changes occur in the 1300–1000-cm^{-1} region with respect to native PUPA. Two bands at 1200 and 1144 cm^{-1} appear as additional positive bands together with the negative peak at 1153 cm^{-1}. Moreover, both the ether C—O—C asymmetric and urethane O=C—O symmetric stretching[10] are shifted to higher frequencies with respect to those in the PUPA spectrum (from 1107 to 1115 cm^{-1} and from 1070 to 1080 cm^{-1}, respectively). This means that in the coated sample some hydrogen bonds between these groups and

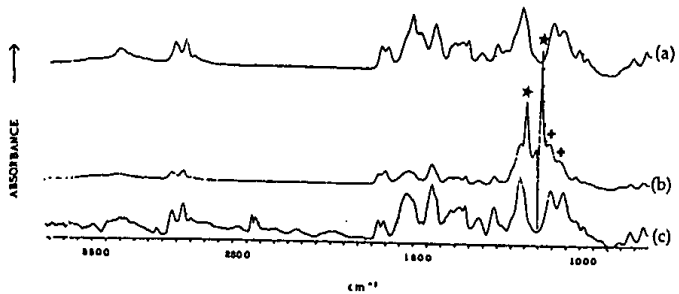


Figure 6. Difference spectra of PUPA-coating on: (a) glass, (b) Gore-tex, (c) Dacron. * bands due to "interacting" C—F group, + bands due to C—O—C asym. and O=C—O sym. stretching of PUPA.

the N—H group, occurring in the PUPA material,[4] are broken because of the preferential interaction of the latter with the more electronegative C—F group of Gore-tex.

This behavior was revealed by the difference spectrum of Gore-tex obtained by subtracting the spectrum of PUPA from that of the coated sample. In Figure 7, the difference spectrum (a) is compared to the spectrum of native Gore-tex (b) in the 1400–1000-cm^{-1} range. Comparison of the two spectra shows that the two C—F absorptions at 1210 and 1153 cm^{-1} of Gore-tex[12] are in part shifted to lower frequencies upon coating. In fact the difference spectrum (a) reveals two strong bands at 1200 and 1144 cm^{-1} and two shoulders at 1210 and 1153 cm^{-1}. Moreover, the ratio of band intensity obtained by summing the heights of the peak at 1144 and the shoulder at 1153 cm^{-1} (due to the "interacting"[13] and "free"[12] C—F stretching, respectively) to that obtained by summing the heights of the peak at 1200 and the shoulder at 1210 cm^{-1} (due to the "interacting"[13] and "free"[12] C—F stretching again) in the difference spectrum, is equivalent to the 1153 cm^{-1}/1210 cm^{-1} intensity peak ratio in the spectrum of native Gore-tex. This emphasizes that some of the C—F groups were involved in a hydrogen-bonding interaction with the coating material. This kind of interaction generally provokes a drop in the absorption frequency of the group involved.[13]

PUPA coating stability investigation

In the case of both coated PVC and PU, a stable coating was formed, due to the penetration feature of PUPA solution in the bulk of the substrate, but in the case of coated glass, Gore-tex, and Dacron, the stability of the PUPA coating was different. In the case of PUPA-coated glass it was found that the layer of coating material can be removed from the substrate after contact with H_2O, DMF, and CH_2Cl_2. The PUPA material swells in these solvents due to the solubility of the poly(amido-amine) in CH_2Cl_2 and H_2O, and of PU in DMF,[4] allowing the detachment of the PUPA coating from the glass surface by scratching. Other solvents, like acetone or toluene, in which the two components of PUPA are insoluble, did not cause this effect. In the case of Gore-

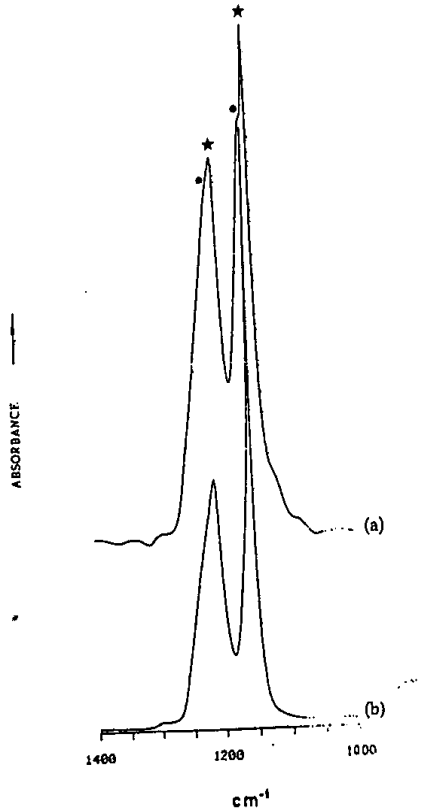


Figure 7. (a) Difference spectrum of Gore-tex obtained from the spectra of PUPA-coated Gore-tex and PUPA, (b) spectrum of native Gore-tex. * bands of native Gore-tex ("free" C—F stretch.), * bands due to "interacting" C—F group.

tex and Dacron, PUPA envelops the fibers and Soxhlet extraction of the coated-samples in CH_3OH/H_2O, was not sufficient to remove the PUPA coating. PUPA coating stability on Gore-tex and Dacron surfaces in flowing saline solution at 37°C revealed that the quantity of PUPA on both sample surfaces remained constant over the period of 1 month, indicating that the layer of PUPA on the substrate is stable at least for this length of time. These results are shown in Table I.

Surface chemical characterization and heparin adsorption

PUPA material has been demonstrated to strongly bind heparin because of the presence of basic aminic nitrogens in its structure. Once protonated,

TABLE I
Stability of PUPA Coating on PVC, PU, Glass, Gore-tex, and Dacron Surfaces under Flowing Saline Solution at 37°C

PUPA-Coated Material	Several days[c]
PVC[a]	∞
PU[a]	∞
Glass	Several days[c]
Gore-tex[b]	≥1 month
Dacron[b]	≥1 month

[a]Penetration occurs with blending of the coating and substrate.
[b]Samples were weighed before and after exposure to the flowing saline solution. No variation in the sample weight was detected within 1 month.
[c]PUPA coating can be removed by scratching.

these may interact with the negatively charged groups of the heparin molecule.[4,14] Two kinds of binding occur between heparinized and PUPA surface. The first seems to be physical adsorption, because heparinized PUPA surfaces release heparin when the samples are placed in a stream of saline solution. The second is a stronger ionic bond between the negatively charged groups of heparin and the protonated basic nitrogens of PUPA.[14] This ionically bound heparin is detached from the surface only with 0.1 M NaOH solution, which deprotonates the PUPA $N-H^+$ groups.

In the case of PUPA-coated PVC, the heparinization was performed using heparin solution in both $H_2O/C_2H_5OH/CH_3COOH$ and H_2O/CH_3COOH because the DOP plasticizer present in the PVC was partially extracted when the sample was bathed in a solution containing alcohol, generating a very rigid material. This extraction was verified by FT-IR. In the spectrum of the sample soaked in the solution containing alcohol some bands due to the DOP plasticizer decreased in intensity with respect to those of polyvinylchloride.

Using the water/acetic-acid solution the coated material did not change its physicochemical properties. The IR spectrum of the sample soaked in H_2O/CH_3COOH solution was the same as that of the native sample and its flexibility unchanged.

The quantities of the stable heparin on the surface of the different coated materials are summarized in Table II. Because of the impossibility of exactly

TABLE II
Quantity of Titrated Nitrogens and Ionically Bound Heparin on PUPA-Coated Materials

PUPA-Coated Material	Titrated Nitrogens	Ionically Bound Heparin[a]	Hep./Titrated N[b] (mg/μmol)
PVC	1.9 μmol/cm²	1.0 mg/cm²	0.5
PU	2.1 μmol/cm²	1.2 mg/cm²	0.6
Glass	0.1 μmol/cm²	0.03 mg/cm²	0.3
Gore-tex	40.7 μmol/g	4.8 mg/g	0.1
Dacron[c]	50.0 μmol/g	5.7 mg/g	0.1

[a]Heparin detached from the surface by 0.1 M NaOH solution.
[b]Heparin/titrated nitrogens in PUPA = 0.7 mg/μmol.
[c]Surface coated with a 30% solution of PUPA in DMF.

measuring the surface area of PUPA-coated Gore-tex and Dacron, the amount of titrated nitrogens and ionically bound heparin were calculated per unit weight of sample.

The heparin/titrated nitrogens ratios, which are independent from the surface or weight of the considered samples, are reported in Table II.

We can note that a higher ratio is obtained for PUPA-coated PU and PVC than for PUPA-coated Dacron and Gore-tex. That difference can be ascribed to the different thickness of "PUPA layer" which covers the substrates. In the case of PU and PVC we obtain a penetration of PUPA until about 350 μm into the bulk (see Fig. 3). In the case of Dacron and Gore-tex we obtain only a superficial layer of PUPA less than 10 μm thick as evidenced by their IR spectra (see Fig. 2), where the bands of the substrates are present together with those of PUPA.

The heparinization process leads to a full protonation of the poly(amido-amine) chains both on the surface and in the bulk of the material, and the high quantity of heparin bound to PUPA surfaces is explained in terms of the high charge density.[4,10] In the cases here considered the charge density is of course higher in PUPA-coated PU and PVC than in PUPA-coated Dacron and Gore-tex because of the thickness of "PUPA layer." This justifies the heparin/titrated nitrogens ratio which is higher for the first two samples than for the other ones. As a matter of fact, in the native PUPA sample obtained from the same solution used to coat these substrates, the heparin/titrated nitrogens ratio is about 0.7 (see Table II).[15]

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15. Unpublished results.

Received May 8, 1990
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Formation of 4,4′-methylenedianiline in polyurethane potting materials by either γ-ray or autoclave sterilization

Hideharu Shintani* and Akitada Nakamura
National Institute of Hygienic Sciences, Department of Medical Devices, 1-18-1, Kamiyoga, Setagaya, Tokyo, Japan 158

In this experiment, we found that a potential carcinogen, 4,4′-methylenedianiline (MDA), was produced from γ-ray irradiated potting materials, medical thermosetting polyurethane (PU). γ-Ray irradiated PUs were immersed in either methanol or in equine serum. The time course of MDA leached from the potting materials and other variables were compared. A significant relationship was noted between the amount extracted and the rigidity of the potting material. The accumulated amount of extracted MDA (a few ppm in the original sample) increased with increasing irradiation dose by a second-order equation. One sample indicated reduced MDA elution after irradiation in the early stage. MDA extraction with serum was 82–87% that with methanol. In one case, MDA leaching into serum in the early stage was higher than that into methanol. Autoclave sterilization did not promote MDA formation, thus indicating that autoclaving would be preferable.

INTRODUCTION

Polyurethane's (PU) properties render it appropriate for a wide range of uses in constructing medical equipment, including potting material for plasma separators, artificial dialyzers, and as a constituent of intra-aortic balloons, ventricular assist devices, and vascular grafts.[1] It is used in a variety of applications due to its compatibility with blood.[1]

γ-Ray sterilization, autoclaving, and chemical sterilization are used to disinfect many of these PU devices. γ-Ray sterilization and autoclaving are thought to cause toxic and low-molecular-weight components to be formed in PU as a result of polymer decomposition.[1] It has been reported that a potential carcinogen, 4,4′-methylenedianiline (MDA, *p,p′*-diaminodiphenylmethane), is formed in thermoplastic PU during autoclave sterilization and is detectable at a level of a few parts per billion upon aqueous extraction.[1–3] The carcinogenic, mutagenic, and toxic properties of MDA and MDA analogs have already been reported.[4–18,24–26]

*To whom correspondence should be addressed.

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FORM APPROVED
O.M.B. NO. 68-R0249

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

GRANT APPLICATION

FOLLOW INSTRUCTIONS CAREFULLY

LEAVE BLANK		
TYPE	ACTIVITY	NUMBER
REVIEW GROUP		FORMERLY
COUNCIL/BOARD (Month, year)		DATE RECEIVED

1. TITLE OF APPLICATION (Do not exceed 56 typewriter spaces)
IONIC/HYDROPHILIC DENSITY:PLATELET/MONOCYTE ADHERENCE

2. RESPONSE TO SPECIFIC PROGRAM ANNOUNCEMENT [X] NO [ ] YES (If "YES," state RFA number and/or announcement title)
New Investigator Research Award

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME (Last, first, middle)
HELMUS, MICHAEL N.

3b. SOCIAL SECURITY NUMBER
192-386-067

3c. MAILING ADDRESS (Street, city, state, zip code)
Franklin Research Center
Physical and Life Sciences Department
20th and Race Streets
Philadelphia, PA 19103

3d. POSITION TITLE
Research Scientist I

3e. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT
Dept. of Physical and Life Sciences

3f. TELEPHONE (Area code, number and extension)
(215) 448-1688

3g. MAJOR SUBDIVISION
Biosciences

4. HUMAN SUBJECTS, DERIVED MATERIALS OR DATA INVOLVED
[ ] NO [X] YES (If "YES," form HEW 596 required)

5. RECOMBINANT DNA RESEARCH SUBJECT TO NIH GUIDELINES
[X] NO [ ] YES

6. DATES OF ENTIRE PROPOSED PROJECT PERIOD (This application)
From: Dec. 1981 Through: Dec. 1984

7. TOTAL DIRECT COSTS REQUESTED FOR PROJECT PERIOD (from page 5)
$ 107,500

8. DIRECT COSTS REQUESTED FOR FIRST 12-MONTH BUDGET PERIOD (from page 4)
$ 37,500

9. PERFORMANCE SITES (Organizations and addresses)
Franklin Research Center
20th and Race Streets
Philadelphia, PA 19103

10. INVENTIONS (Competing continuation application only)
Were any inventions conceived or reduced to practice during the course of the project?
[ ] NO [ ] YES - Previously reported
[ ] YES - Not previously reported

11. APPLICANT ORGANIZATION (Name, address, and congressional district)
Franklin Research Center
20th and Race Streets
Philadelphia, PA 19103

12. ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR INSTITUTIONAL GRANT (See instructions)
Code [6 0] Description:

13. ENTITY IDENTIFICATION NUMBER
23-1307050?

14. TYPE OF ORGANIZATION (See instructions)
[X] Private Nonprofit
[ ] Public (Specify Federal, State, Local):

15. OFFICIAL IN BUSINESS OFFICE TO BE NOTIFIED IF AN AWARD IS MADE (Name, title, address and telephone number)
Frank T. Robinson
Contract Administrator
Franklin Research Center
20th and The Parkway
Philadelphia, PA 19103 215-448-1406

16. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION (Name, title, address and telephone number)
John R. Stover
Vice President
The Franklin Research Center
20th and The Parkway
Philadelphia, PA 19103 215-448-1219

17. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PERSON NAMED IN 3a (In ink. "Per" signature not acceptable)

DATE

18. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense. (U.S. Code, Title 18, Section 1001.)

SIGNATURE OF PERSON NAMED IN 16 (In ink. "Per" signature not acceptable)

DATE

PHS 398
Rev. 10-79

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE

ABSTRACT OF RESEARCH PLAN

LEAVE BLANK

PROJECT NUMBER

NAME AND ADDRESS OF APPLICANT ORGANIZATION (Same as Item 11, page 1)

FRANKLIN RESEARCH CENTER, 20th and Race Streets, Philadelphia, PA 19103

TITLE OF APPLICATION (Same as Item 1, page 1)

IONIC/HYDROPHILIC DENSITY:PLATELET/MONOCYTE ADHERENCE

Name, Title and Department of all professional personnel engaged on project, beginning with Principal Investigator/Program Director

Michael N. Helmus, Ph.D., Research Chemist I, Department of Physical and Life Sciences

ABSTRACT OF RESEARCH PLAN: Concisely describe the application's specific aims, methodology and long-term objectives, making reference to the scientific disciplines involved and the health-relatedness of the project. The abstract should be self-contained so that it can serve as a succinct and accurate description of the application when separated from it. DO NOT EXCEED THE SPACE PROVIDED.

The main objective is to examine the role of surface charge hydrophilicity and spatial distribution of the anionic and hydrophilic groups on human platelet and monocyte adhesion and function. The platelet will be used as a model for a cell intimately involved in thrombus formation while the monocyte will be used as a model of phagocytic cell involved in thrombus resolution. Platelet function will be gauged by serotonin release (an indication of their propensity to aggregate) and monocyte function will be gauged by chemiluminescence (an indication of the activation of the phagocytic system). The research is designed to test on model substrates, the hypothesis that an optimum degree of either ionic or hydrophilic (polar) interaction is desirable for decreased cell adhesion and activation. The substrates will be a block or random copolymer containing butadiene as one of the components. The butadiene can be modified to contain either anionic moeities or hydrophilic moeities. The order and shape of the molecular domains can be varied by the casting conditions of the block copolymer. The random copolymer will test a homogeneous surface of the same nominal composition. Protein interactions will be observed by suspending cells in deficient plasma and by antibody tagging of adsorbed proteins.

LABORATORY ANIMALS INVOLVED. Identify by common names. If none, state "none"

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR: Michael N. Helmus

TABLE OF CONTENTS

Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 5a, 5b. Type the name of the Principal Investigator/Program Director at the top of each printed page and each continuation page.

Application Receipt Record, form PHS 3820
Form HEW 596 if Item 4, page 1, is checked "YES"

Ionic/Hydrophilic Density: Platelet/Monocyte Adherence

SPECIFIC AIMS:

The main objective is to examine the role of surface charge, hydropholicity, and spatial distribution of ionic (COO- and SO_3-) and hydrophilic (OH) groups on human platelet and monocyte adhesion, and protein interaction. This will be accomplished by the following:

- The preparation of a set of well characterized substrates with respect to surface charge, hydrophilicity and spatial distribution of ionic and hydrophilic groups will be performed. The substrates will be a random and block copolymer of styrene and butadiene. The random copolymer will provide a homogeneous surface while the block copolymer will be processed to produce surfaces having controlled heterogeneity due to the molecular phase separation of the two blocks [1) spherical butadiene and 2) irregular shaped butadienes domains]. The ionic and hydrophilic groups will be isolated on the butadiene component. The substrate will be characterized for domain structure by transmission electron microscopy; hydrophilic and ionic character by contact angles and ionic character by streaming potentials.

- The effect of the material substrates on cellular function will be determined by platelet adherence and serotonin release, and monocyte adherence and chemiluminescence. Cell morphology will be investigated using light microscopy and transmission electron microscopy. The degree of change in morphology from the circulating cells will be used as an indicator of cell-surface interaction.

- The role of plasma proteins on cell adhesion will be investigated by comparing adhesion of the cells suspended in plasma to the cells suspended in buffer, defibrinogenated plasma, and FXII, FXI and prekallikrein depleted plasma. The interaction of γ-globulins and fibronectin with the surface will be examined by using peroxidase-antiperoxidase tagged antibodies to these proteins.

- The interaction of platelets and monocytes from mixed suspensions as evidenced by adhesion and function will be evaluated on the characterized polymeric substrates.

BACKGROUND AND SIGNIFICANCE

A. Introduction and Rationale:

The ability to "design" materials for use in blood contacting devices, requires a knowledge of how a surface interacts with blood. The interaction is complex with many competing reactions, e.g. protein adsorption and

desorption, FXII activation, complement activation, platelet adhesion and activation, platelet aggregation, white cell chemotaxis and adhesion, fibrin formation, fibrinolysis, white cell phagocytosis, and thrombus resolution (1-7). An occlusive thrombus may form if platelet aggregation and fibrin formation occurs rapidly due to the self amplification mechanism of thrombus formation. However, the elaboration of antithrombogenic agents, e.g. heparin, and fibrinolytic enzymes, e.g. plasmin, early in the blood interaction can limit thrombus growth.

The rationale for this research is to study in detail the effect of surface charge, hydrophilicity, and spatial distribution of the ionic and hydrophilic groups on the interaction of two cells, one important in thrombus formation, the blood platelet, and the other in thrombus resolution, the blood monocyte. An *in vitro* experiment is proposed in order to minimize numerous uncontrollable variables. An understanding of the parameters that affect the interaction of these two cells on synthetic substrates will help in the interpretation of future *in vitro* experiments with non-anticoagulated human blood and *in vivo* animal models.

The basis for examining surface charge, hydrophilicity, and molecular domain formation in block copolymers, arises from the increasing experimental evidence of the influence of these parameters on cell adhesion and thrombus formation. The lack of well characterized substrates has been a major difficulty in interpreting past experiments and that this proposed research hopes to overcome. The research is designed to test, with model substrates, the hypothesis that an optimum degree of either ionic or hydrophilic (polar) interaction is desirable for decreased cell adhesion and activation. Furthermore, the presence of moleuclar domains in the polymeric substrate may further modify this response, and this effect will be further examined by isolating the ionic/or hydrophilic groups to these domains. This optimum may not be the same for platelets and monocytes. Furthermore, the optimum may also vary for the number adhered and the measured cell function (e.g. serotonin release in platelets). One major defect in past research has been the arbitrary manner in which surface charge and hydrophilic content has been measured. Surfaces will be characterized with respect to surface charge by streaming potential using standard buffers, hydrophilicity by the advancing and receding contact angles of water and standard buffers of different pH, and bulk hydration, surface polar and dispersion content by contact angles of organic liquids, and molecular domain formation by transmission electron microscopy. These experiments will provide a basis for future research that will evaluate thrombus formation *in vitro* with non-anticoagulated human blood and *in vivo* animal models.

Pertinent Literature Background

a) Platelets

Platelets play an essential role in hemostasis and the interaction with foreign materials. The initial interaction of the platelet with the surface

of a material may determine whether platelet aggregation will occur. Generally, though not always, platelet spreading on a surface is a precursor to the release reaction e.g., serotonin and ADP, followed by aggregation (8-9). Factors influencing platelet interactions include (12,13) e.g. 1) fibrinogen adsorption which generally increases platelet adherence, 2) γ-globulin adsorption which tends to increase the platelet release reaction, and 3) albumin adsorption which decreases platelet adherence.

b) Leukocytes

Recent work (5-7, 14-16) now suggests the white cell plays a significant role in thrombosis. Monocytes appear to play a anti-thrombogenic role as observed by their spreading on the surfaces of non-occlusive thrombus in dogs (5), their presence on surfaces which did not form gross thrombus in dogs (7), their presence prior to endothelialization (5,7) and their potential (though controversial) for transforming into endothelium (17-23). The adherence of leukocytes has, in some cases, caused the detachment of individually adhered platelets (13). The presence of granulocytes appears to enhance platelet aggregation (10,11, 15), as observed by their presence basal to large platelet aggregates. However, both granulocytes and monocytes have the potential to elaborate anti-thrombogenic and fibrinolytic factors while granulocytes may also release a platelet aggregating factor (24-28). Granulocytes and monocytes are intimately involved in phagocytizing cellular debris, with the monocytes transforming into macrophages and potentially fibroblasts (1,17). Adsorption of fibronectin and γ-globulin appears to increase leukocyte adhesion (15,29-31).

Surface Properties

The properties of a surface are ellusive and difficult to define (32). Many of the surface characteristics which have been used--critical surface tension, surface polarity, total surface tension and surface charge--average the surface proterties, and ignore molecular heterogenity (2, 5, 32). Therefore, the proposed research will vary these properties with surfaces having controlled degrees of heterogeneity.

Hydorphilic surfaces have generally demonstrated decreased thrombus formation and cellular achesion (5,33). However, such surfaces may also show increased platelet consumption (21,34-35) as evidenced by a decreased *in vivo* lifetime of circulating platelets. Some hydrophilic polyurethanes showed decreased platelet adhesion compared to more hydrophilic surfaces, but the platelets that do adhere release serotonin, an aggregating agent of platelets (36).

Negatively charged surfaces generally have shown decreased platelet adhesion (37,39), increased clotting times (40) and decreased thrombus formation (7, 41-44). The neutralization of acid groups with Na^+, K^+, Li^+, and Ca^+ decreased thrombosis (42,45) and Na^+ increased leukocyte adhesion *in vitro* (46). However, not all materials containing negative

surfaces appear thromboresistant (42,47-48). In fact, glass a known activator of FXII, is negatively charged and thrombogenic. Not only the charge density, but the sterographic relationship of the charge may be important. The activation of FXII requires fixed spacing of charges as observed by its activation by homocystine while, it is not activated by cystine (49). Furthermore, as ionization is increased, bulk swelling may occur in some ionic polymers and in some cases effectively dilute the surface charge density and increase thrombosis (41).

The effect of protein adsorption and activation of blood enzymes and subsequent cellular interactions is just coming to the state of understanding. The activation of the intrinsic coagulation sequence alone leads to a bewildering array of interactions and the evolution of chemotactic agents (phasmin, kallikrein, and complement (C5a, C(5,6,7)a, and split C3 fragments) which can influence leukocyte and platelet interactions (13,50-52). Therefore, a portion of the research will examine the role of protein interactions by using plasma, defibrinogenated plasma, buffer, FXII depleted plasma and antibody tagging to adsorbed fibronectin and -globulins (15,30).

The blood interaction is also influenced by the formation of molecular domains in the polymeric substrate. These polymers consist of two chains that are linked together and that are incompatible (i.e. phase separate). In a 30 wt % styrene, butadiene block copolymer, increased perfection of the domains was found to increase platelet adhesion in vitro (2). Similar observations have been observed in the polyurethanes (4), a block copolymer of poly (-benzyl-L-glutamate)-(butadiene/acrylonitril)-(-benzyl-L-glutamate) (53) while a random structure of styrene-HEMA showed a greater degree of platelet adhesion and aggregation than the ordered structure (54).

Based on these observation the proposed research will investigate the role of hydrophilic and ionic density and the effect of their isolation on the phase separated domains. The basic polymer to be studied will be a block copolymer of styrene and butadiene with a composition of 76 wt % styrene. At this composition the polymer will be processed to exhibit a structure that will be butadiene spheres in a styrene matrix (55,55a,56). Rapid evaporation of the casting solvent will produce irregular shaped domains, a non-equilibrium structure (2). The butadiene domains will be modified to have hydrophilic or ionic groups as described in the methods section. A random copolymer of styrene/butadiene, will therefore be studied to provide a homogenous surface of the same nominal composition as the block copolymer. The reaction to produce negative surface sites (See Methods Section) was carried out by Bantjes on isoprene containing polymers in order to produce synthetic heparinoids containing $-COO^-$ and SO_3^- groups (57-58). These polymers exhibited long clotting times, decreased platelet adhesion and binding to antithrombin III.

Future studies will undertake the examination of these model surfaces with whole human blood and in vivo animal studies.

Applicants Relevant Background - Preliminary Studies

I. Publications:

A. Helmus, M. N., O. P. Malhotra and D. F. Gibbons, "Plasma Interaction on Block Copolymers as Determined by Platelet Adhesion", presented at the American Chemical Society conference Biomaterials, Chicago, Ill., Nov. 1980, to be published Advances in Chemistry Series.

B. Helmus, M.N., D. F. Gibbons, and R. D. Jones, "The Effect of Surface Charge on Thrombosis", World Biomaterials Congress, Vienna, Austria, April 1980; In preparation for publication.

Helmus, M.N., D. F. Gibbons, and R. D. Jones, "Surface Analysis of Copolymers of L-Glutamic Acid and L- Leucine", In preparation for publication.

C. Picha, G., M. Helmus, S. Barenberg, D. Gibbons, R. Martin, and Y. Nose, "The Characterization of Intima Development in Left Ventricular Assist Device and Total Artificial Heart", Trans. Amer. Soc. Art. Int. Organs, 22:554 (1976).

Nose, Y, R. Kiraly, G. Jacobs, I. Koshino, N. Morihnaga, S. Kausai, T. Washizu, Y. Mitamcra, K. Nakiri, R. Sukulac, H. Krambic, J. Snow, D. Gibbons, O. Sudilovsky, G. Picha, M. Helmus. "Development and Evaluation of Cardiac Prothesis", Department of Artificial Organs, Cleveland Clinic Foundation, Devices and Technology Branch, Division of Heart and Vascular Diseases, NOI-HV-4-2960-2, March 1976, Progress Report.

Harasaki, H, G., Picha, M. Helmus, A. Fields, R. Kilraly, Y. Nose, "Ultrastructural Study on In Vivo Blood Compatiblity of Biolized Materials," Thrombosis and Haemostasis, Phila., 1977, Poster Session.

Harasaki, H. G., Picha, S. Barenberg, M. Helmus, A. Kiraly, Y. Nose, "Endothelialization of Cardiac Prothesis after Long-Term Implantation", Transactions American Society of Artificial Internal Organs, XII, 1977, Abstract.

II. Previous Work:

The principal investigator has processed and characterized block and random copolymers and studied their behavior in vitro and in vivo. Characterization techniques included contact angles (surface tension and polarity, and hysteresis), transmissing electron microscopy, streaming potentials, differential scanning colorimetry, x-ray diffractometry, bulk hydration, scanning electron microscopy, and Nomarski differential interference contrast microscopy. Biological evaluations included light, scanning electron, and transmission electron microscopy, and standard histological sections.

A. In one study, a series of block copolymers, with controllable domain morphology, were tested to determine the effect of surface wettability, morphology, and chemistry on the attachment of gel filtered human platelets. The surfaces were first exposed to a plasma for 3 sec or 3 min and then to platelets, suspended in Tyrode's buffer in 0.35% albumin (pH 7.4). The most hydrophobic surface, styrene-butadiene-styrene (SBS) (30 wt % styrene) attached the most platelets followed by the less hydrophobic polyurethane, and lastly the hydrophilic polystyrene-polyethylene oxide (PS-PEO) which attached essentially none. Phase separation in polyurethane and in SBS significantly (P 0.025) increased the adherence of platelets after exposure to PPP for 3 sec and 3 min, respectively. No such difference was observed in PS-PEO. The SBS, with and without long range order attached significantly (P 0.025) more platelets at 3 sec than at 3 min. Neglible platelet adhesion occurred when heat defibrinogenated plasma was exposed to the surface.

It appears that the SBS block copolymer, as compared with hydrophobic glass, adsorbs fibrinogen loosely but more tightly than hydrophilic glass. Phase separation causes the protein to attach more strongly (See Appendix).

B. In a separate study (7) the role of surface charge in arterial thrombus formation in dogs was studied on implants in the femoral and carotid arteries of dogs. The implants were formed from copolymers of L-glutamic acid co-L-Leucine. Implants were pre-equilibrated in steril TRIS-buffers of different pH prior to implantation. Thrombus formation at 2 hours of implantation was found to correlate with the initial surface charge as determined by streaming potentials. The percentage of the implant surface covered by thrombus decreased as the initial surface charge increased and was essentially zero when 10% of all available surfaces sites were ionized. However, if more than 10% of the available surface sites were un-ionized glutamic acid, a large area of surface was covered by thrombus regardless of the initial degree of ionized sites. At longer implantation times (4 & 7 days), the formation of endothelial-like cells was enhanced on thrombus covered surfaces.

A unique analysis of the surface of the copolymers was performed by measuring the advancing and receding contact angles of buffers of different pH. This provided a technique to measure the chemical heterogenecity of the surface. The advancing angle remained constant as a function of pH but the receding angle decreased in a fashion similar to a titration curve. This method provides a method of characterizing the hydrophilic and ionization characteristic of surfaces containing acid or base groups (See Appendix).

C. In a third study, thrombus formation in total artificial hearts and left ventricular assist devices was studied. The degree of thrombus formation was a function of flow patterns and areas of stasis. When the pumping bladder (hexsyn rubber) was covered with gelatin coated dacron [the gelatin cross-linked with gluteralhyde] a large degree of thrombus was formed. However, minimal thrombus formed on a gelatin coated hexsyn rubber bladder except when cracks formed in the gelatin. A five year passive implant in a

steer showed a thrombus free surface, except for cellular attachment, on the gluteraldehyde fixed pericardium used to line the housing (See Appendix).

III. Preliminary Work:

Initial studies carried out by the applicant indicate that the reactions necessary to modify the styrene-butadiene-styrene (SBS) rubber are feasible. The reaction to produce anionic groups on the butadiene phase was carried out on a film of cast SBS using the reaction described by Bantjes in the following section. The effect of the modification can be seen by the contact angle of water on the surface. The untreated SBS has an advancing angle of about 93°. However, the treated surface is more wettable with an advancing angle of only 30°, indicating the presence of a wettable component.

METHODS

I. Experimental Design

The research will examine the role of hydrophilicity, charge, and the spacing of the hydrophilic and ionic groups on platelets and monocyte adhesion and protein interaction. The substrate to be studied will be a block and random copolymer of styrene and butadiene. The equilibrium structure of domains in the block copolymer will be butadiene spheres (about 200 angstroms in diameter) in a matrix of styrene. Irregular shaped domains will be produced by increasing the evaporation rate of the casting solvent while a homogeneous surface of the same composition will be obtained by using a random copolymer. The effects of charge and hydrophilicity will be observed by 1) varying the surface concentration of the hydrophilic (OH) and ionic groups (SO_3^- and COO^-) and 2) observing the effect of spatial distribution by isolating these groups on the butadiene phase. A surface of 100% styrene will be used as a control apolar surface while NaOH cleaned glass will be a control surface that causes activation of FXII.

The platelet will be used as the model for the cell intimately involved in thrombus formation. Cell adhesion will be studied in a Mason cell (59) in order to eliminate the air interface. The number adhered will be determined while their propensity to aggregate will be evaluated by serotonin release (31,40). These two parameters will be correlated to platelet morphology by light and transmission electron microscopy (TEM).

The monocyte will be used as the model of the phagocytic cell involved in thrombus resolution. The number adhered and morphology will be determined using a Mason cell. The activation of the phagocytic system will be gauged by chemiluminescence (60). Interactive effects of platelets and monocytes will be determined by mixed suspension of both cell types.

The role of various plasma proteins will be gauged by comparing adhesion in plasma to adhesion in 1) buffer, (a protein free media in order to observe

the physical effects of the surface on the cells) 2) defibrinogenated plasma (to observe differences in cell adhesion in a fibrinogen free media) and 3) in exhausted plasma (FXII, FXI, prekallikrein depleted plasma) in order to observe the role of the intrinsic coagulation system on cell adhesion. Activation of FXII can lead to the evolution of chemotactic agents to leukocytes (complement, Kallikrein and plasmin). Antibody tagging (by the Peroxidase-anti peroxidase method) of adsorbed plasma proteins (e.g. γ-globulins and fibronectin) will be under taken to observe their concentration on the substrates and any correlation to the cellular interaction (15).

As the development and characterization of the ionic, hydrophilic, and domain structure of the polymers is proceeding, initial biological testing of the cellular adhesion will begin on the control surfaces (100% styrene and NaOH cleared glass) and the sytrene-butadiene random copolymer. The effort during the first year will be development of the copolymer surfaces, the cell adhesion from plasma, and cell morphology as determined by light microscopy. During the second year the effort will shift to cellular function (platelet serotonin release) and monocyte chemiliminescence) and cell adhesion from buffer, defibrinogenated plasma, and exhausted (FXII, FXI, and prekallikrein depleted) plasma. During the third year emphasis will shift to protein interactions as determined by Peroxidase anti-Peroxidase tagged antibodies to fibronectin and γ-globulins.

II. Techniques

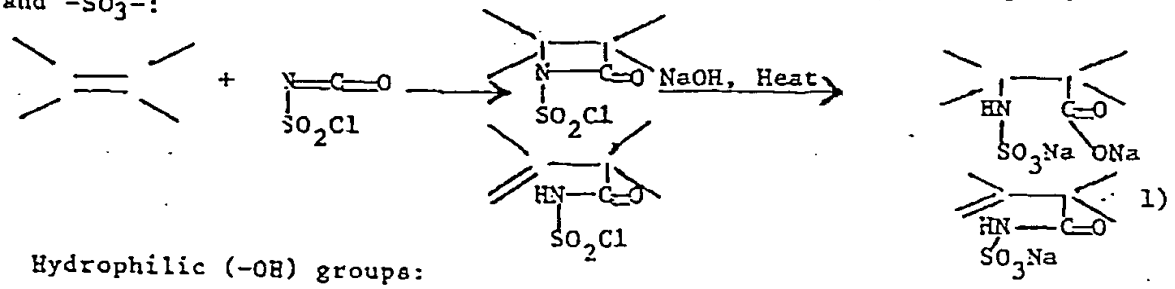
1. Polymer Processing and Modifications

a. The polymer will be either a random or block copolymer of 76 wt % styrene and 24 wt % butadiene (Phillips Chemical Co. K103 or Solprene 314). The random copolymer will produce a homogeneous surface without phase separation. The molecular phase separation of the block copolymer can be controlled by the casting solvent and the evaporation rate of the solvent (2). The use of a polar solvent preferential for the styrene phase, e.g. methyl ethyl keytone, and a slow evaporation rate will favor an equilibrium structure that will consist of butadiene spheres (about 200 A° in diameter) in a matrix of styrene (55a). Rapid evaporation of the casting solvent will produce domains of irregular shape (2). A sample of 100% styrene will be used as a control apolar surface. NaOH cleaned glass will be used as a control surface that is known in to activate the intrinsic coagulation cascade.

b. Surface modifications will be performed using techniques described in detail in the literature (57-58, 51-55). Reactions will be performed on the cast samples with the reactive reagents in a solvent that will not dissolve the cast polymer. The cast polymer surfaces will be reacted such that 33, 66, and 100% of the exposed butadiene is reacted. The optimum concentrations and times of the reactions need to be determined.

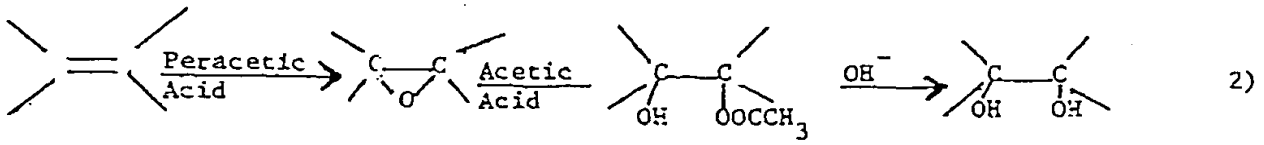
a) Anionic Groups Groups ($-COO^-$ and $-SO_3-$)

These groups are produced by the reaction of the diene component of butadiene with chlorosulfonyl chloride (57-58, 61-63). The chlorosulfonyl isocyanate will be dissolved in hexane and only the surface of the substrate will be reacted. After rinsing the surface with hexane and drying, the surface will be placed in a solution of NaOH at 100-110°C to produce groups of $-COO^-$ and $-SO_3-$:



b Hydrophilic (-OH) groups:

The reaction as described by Sefton and Merrill (64,65) produces the -OH group on the diene portion of Butadiene by reaction with peracetic acid and acetic acid, followed by sodium hydroxide:



2. Polymer Characterization

a. Surface charge per unit area will be determined from streaming potentials using the procedure as described by Van Wagenen (66) and the calculations as described by the applicant (7), as based on the Helmholtz and Smoluchowski equation, the Boltzman distribution of ions, and applying Poissons equation (67,68). By using buffers of constant ionic strength and varying the pH, the ionization characteristics of the surface can be examined, including the pK.

b. Contact Angles will be used to determine wettability and the ionization characteristics of the surface by using buffers of different pH (as described in the section on Previous Work). Measurement of the advancing and receding angles is critical to determine chemical heterogeneties on the surface (69). When these heterogeneties are on the order of 0.1 microns or larger, the advancing angle is sensitive to the hydrophobic component of the surface while the receding is sensitive to the wettable complement (69).

Measurment of contact angles using organic liquids will be used to give a measure of the polar and dispersive nature of the surface and the critical surface tension. Again measurement of the advancing and receding angles are important (2,7).

c. Bulk swelling will be determined in water, buffers, and serum to give an indication of hydration, the ionization characteristics, and the effect of proteins and a high osmolarity (serum) on hydration. If swelling in a physiologic environment is significant the biological interactions may be influenced by bulk water and ion gradients, if the samples are not preequilibrated under physiologic conditions (7).

d. Mass Spectroscopy will be used to characterize the chemical moieties produced by the surface modifications. However, these will be bulk measurements and will not be significant in determining surface concentrations of the groups.

e. Transmission electron microscopy will be performed on thin films (about 500 A° thick) and microtomed sections that have been stained with O_sO_4 (preferential for butadiene) in order to determine domain morphology (2). Samples that are only partially reacted to produce the anionic and hydrophilic groups should also stain. However, it would not be expected that the domain shape would change by these reactions since the butadiene is constrained by the surrounding styrene matrix.

2. Methods for Biological Materials and Assays

a. Human platelet rich and Poor Plasma (2):

Blood will be drawn by venipuncture into 25 cc syringes and be anticoagulated wil 0.11 M sodium citrate in a 9:1 ratio. All donors will fast overnight and will not have taken medication for at least two weeks. The first 3 or 4 cc of blood will be discarded prior to further blood collection in order to eliminate contamination of the blood from tissue fluids. Platelet rich plasma (PRP) will be prepared by placing the anticoagulated blood in polypropylene tubes and centrifuging at 77 g for ten minutes at room temperature. Platelet poor plasma (PPP) will be prepared by centrifuging the blood at 23,000 g for 10'.

b. Deficient Plasma:

Plasma will be defibrinogenated by heating at 56°C for 10 minutes. Fibrinogen will precipitate out of solution. FXII, FXI, and prekallikrein deficient plasma will be prepared as described by Ratnoff (70):

> Normal blood is collected in one-fiftieth volume of 0.50 M sodium citrate buffer (pH 5.0) in thin walled polypropylene tubes and centrifuged at 2°C for 15 min at 3500 rpm. The plasma is then put in a polypropylene tube and adsorbed with Johns Manville Filter-Cel Celite, 15 mg per ml, at room temperature for 5 min, mixing continuously with a magnetic stirrer. The mixture is centrifuged in thin-walled polypropylene tubes at 2°C for 10 min at 3500 rpm. The adsorption is then repeated. The plasma is then incubated in stoppered thin-walled polypropylene tubes at 37°C for 24 hrs. The exhausted plasma is then stored at -20°C.

c. Platelet Suspension (3)

After PRP is formed, it will be spun at 1000 g for 10 minutes at room temperature to form a platelet button. Artificial medium (140mM NaCl, 5mM KCl, 5mM glucose, 1/20 volume of acid citrate anticoagulant, pH 6.5 at 37°C) will be added to the pellet and the platelets will be resuspended by gently tilting the tube. The process will be repeated and the pH brought to 7.4 with calcium free Tyrode's buffer.

Platelets will also be resuspended in the defibrinogenated and exhausted plasmas. A control for the effect of resuspending the platelets will be testing resuspended platelets, in plasma with the original platelet rich plasma. Platelet concentrations will be determined by a Coulter counter.

d. Human Monocytes (71)

Preparations of human peripheral blood mononuclear leukocytes are obtained from Ficoll-Hypaque gradients as described by Snyderman: heparinized (10 microliters/ml) venous blood is diluted 1:4 in physiological saline and approximately 35 ml placed in Falcon No. 2070 conical centrifuge tubes. Twelve milliliters of a mixture containing 2.4 parts of 9% Ficoll (Pharmacia Fine Chemicals, Piscataway, New Jersey 08854) and 1 part 33.9% hypaque (Winthrop Laboratories, Atlanta, Georgia 30304) is injected below the diluted blood using a 50-ml syringe and a 16-gauge, 4-inch spinal needle. Following centrifugation at 20°C for 40 min at 400 g, the diluted plasma is aspirated and the buffy coat removed, using an inch siliconized Pasteur pipette. A maximum of 7.5 ml of the cell suspension is placed in 50-ml Falcon centrifuge tubes and to this is added 40 ml of phosphate buffer (pH 7.0) isotonic saline containing 0.1% gelatin (PBS). The tubes are centrifuged for 15 min at 300 g 4°C. The cell pellets are washed once more with PBS and then are combined, resuspended in plasma and the deficient plasmas. Monocyte concentration will be determined by counting in a Hemocytometer.

e. Assay for Cell Adhesion:

Cell adhesion will be performed in a cell similar to that described by Mason (59). The material under study is coated on microscope slides and treated as desired. These slides sandwhich a silicon-rubber gasket that contains an ellipitcal cut-out and holds a volume of 2 cc. An inlet and outlet are produced by piercing the two ends of the chamber with silicone-coated 18 gauge needles. Prior to testing the cell is filled with physiologic saline to eliminate the air interface. Cell suspensions are drawn into the chamber and remain in contact with the surfaces for 10 minutes at 37°C. Test cells are rotated every 1 1/2 minutes to eliminate settling effects (4).

The suspensions will be rinsed out of the test cell and adhered cells will be fixed with gluteraldehyde buffer. Samples will be dehydrated in increasing concentrations of acetone/water and then Freon TF/acetone (7). Samples to be counted by reflected light Nomarski microscopy will be air

dried. Any samples for SEM will be critical point dried. Samples for TEM will be fixed in OsO_4/gluteraldehyde solution, dehydrated, embedded in epon, sectioned for TEM, and stained with uranyl acetate. The number of adhered cells will be determined by counting cells in at least 10 random fields of view at a magnification of 1000 x (2,7). For platelets, cellular morphology will be described as 1) discoid--same shape as in circulating blood 2) dendritic with central core and either a) short psuedopods or b) long psuedopods and 3) spread platelets defined by a thin, broad, spread membrane. Monocyte morphology will be described in a similar fashion 1) round-same as in circulating blood 2) long or short psuedopod formation and 3) spread.

Statistical analysis of adhered cells will be performed by analysis of variance (2,7).

f. TEM sections will compare cell morphology to those of cells fixed in suspension--the reorganization of organelles or the absence of specific organells (e.g. the dense granual of platelet that stores serotonin).

g. Platelet Serotonin Release

Platelets are prepared as described by Brash et al (40,33):

The platelets are separated by centrifugation, washed three times and suspended in Tyrode solution containing albumin (0.35 g/100 ml) and apyrase to degrade ADP and prevent platelet aggregation. The platelets are labelled in the first wash by incubating with ^{51}Cr-Na_2CrO_4 and ^{14}C-serotonin for 45 min at 37°C and suspended at the desired platelet count in Tyrodes buffer, plasma or deficient plasmas.

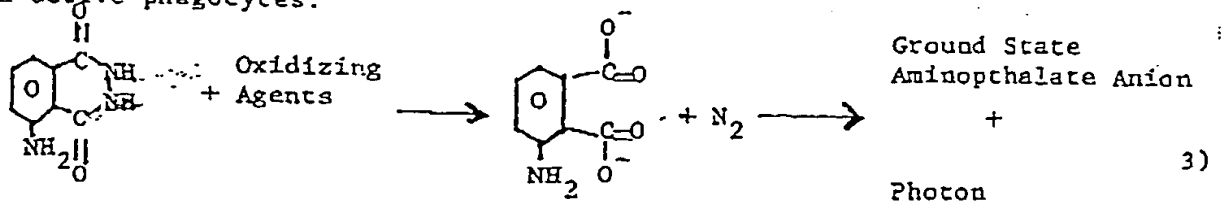
After the adhesion assay the adhered platelets are rinsed with buffer to remove platelets from the surface for counting, the rinsed specimens are placed for 2 hours in counting vials each containing 10 ml of a scintillation cocktail. The solid specimens are then removed and the liquid extract will be counted for both ^{51}Cr and ^{14}C in a automatic scintillation analyzer.

Adhesion and release are determined by comparing the radioactivity of the surface with that of an aliquot of suspension of known platelet concentration. In the absence of demonstrable platelet lysis the surface count of ^{51}Cr can be used to compute the number of platelets adhering per unit area when compared to the suspension count. The surface count of ^{14}C when compared to the ^{51}Cr surface count and the to the solution ^{14}C count, will show a proportionate decrease if release of serotonin has accompanied adhesion.

h. Chemiluminescence (60).

This assay will be performed on monocytes adhered to the polymeric substrate coated on liquid scintillation (LSC) vials. A luminol-amplified

system will be employed. Luminal demonstrates chemiluminescence upon reacting with the oxidizing agents (e.g. O_2^-, H_2O_2, $\cdot OH$, O_2') that are present in active phagocytes.



3)

The luminol will be made water soluble by using 1.42 g luminol, 78 g potassium hydroxide, and 61.8 g boric acid in 1 liter of solution to prepare an 8 mM stock solution of luminol. Some initial effort will be needed to determine the optional amount of luminol to be added for the assay. The amount of light emitted will be determine using a Liquid Scintillation Counter set with the parameters as described by Trush et al. (60) (Table I):

i. Protein Interactions:

The interaction of globulins and fibronectin on the substrates will be determined by Peroxidase-Antiperoxidase antibody tagging (15,72,73). Antihuman -globulin and fibronectin (Collaborative Research) are available commercially. The assay will be performed in the Mason Cell (59) using citrated plasma, defibrinogenated plasma, or exhausted plasma. The surfaces will be exposed to the plasma for ten minutes, rinsed and fixed in 1.0% glutaraldehyde, dehydrated, embedded in epon and thin sectioned. Sections on nickel grids will be floated on drops of the desired reagents and staining solutions.

TABLE I. (60)

LIQUID SCINTILLATION COUNTER (LSC) SETTINGS USED TO MEASURE PHAGOCYTOSIS-ASSOCIATED CHEMILUMINESCENCE

1. Present time	1 Minute
2. Counting channel (linear 0-60 to 1000 divisions)	Width 5 divisions Lower discriminator A, 0-60 Upper discriminator B, 1000
3. Gain	60-100%
4. Coincidence	Off
5. Refrigeration	For optimal activity, turn off for at least 24 hr prior to use
6. Input selector (photomultiplier tube switch)	1 + 2

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Complement Activation in Extracorporeal Circuits

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INTRODUCTION

Complement activation reactions normally make an important contribution to host defense mechanisms by promoting localized generation of acute inflammatory mediators. However, it is now apparent that blood contact with many types of biomaterials initiates complement activation and, in the case of extracorporeal medical devices, fosters systemic production of bioactive molecules that can provoke specific types of clinical sequelae. Consequently, biomaterial scientists have begun to emphasize delineation of the complement-activating potential of blood-contacting materials employed in the manufacture of medical devices. For example, our previous investigations have centered on both laboratory and clinical evaluation of hemodialyzers and cardiopulmonary bypass oxygenators.[1] In particular, studies aimed at characterizing the complement-activating potential of hemodialyzers have been extremely useful for defining the membrane or material characteristics that regulate clinical exposure to the human anaphylatoxins as well as for delineating the pathophysiologic responses that result from systemic complement activation.[2-4] Before discussing and interpreting these specific findings, it will be helpful to summarize briefly our current understanding of the mechanisms of complement activation and the biological properties of the human anaphylatoxins.

COMPLEMENT ACTIVATION

Alternative Pathway Mechanisms

It is generally accepted that the complement activation observed during hemodialysis proceeds by alternative pathway mechanisms. The proteins and biochemical events that compose the alternative pathway have previously been described in detail[5] and may be briefly summarized as follows.

Activation of the alternative pathway involves four distinct phases: initiation, deposition of C3b, recognition, and amplification. The first phase, initiation, begins in plasma and involves spontaneous formation of a short-lived intermediate termed metastable C3b. The active site of this reactive species consists of an activated thiolester that can undergo nucleophilic attack (FIGURE 1). Consequently, this reactive form of C3 can deposit on and become covalently attached to biomaterials or other surfaces that possess nucleophilic characteristics. The second step, recognition, involves discrimination between materials that serve as activators or nonactivators. This process of discrimination occurs very soon after C3b becomes attached to the surface and essentially results from regulation of the binding of the inhibitory protein factor H to the surface-bound C3b. For example, materials that behave as activators of the alternative pathway tend to limit binding of factor H to C3b and favor assembly of

the positive regulatory components, factors B, D, and P. When this occurs, C3b in the presence of factors B and D and Mg^{2+} forms a C3 convertase capable of generating many molecules of C3b that are identical to the original C3b that was initially deposited on the surface. In this way, amplification of the signal through a positive-feedback mechanism takes place and rapid assembly of the C3 and C5 convertase enzymes is achieved. By contrast, some materials behave as nonactivators of the alternative pathway. In these cases, factor H binding to surface-bound C3b is greatly enhanced when compared to that of factor B. Thus, nonactivating surfaces are characterized by the fact that they promote catabolic inactivation of C3b and termination of the amplification feedback mechanism.

The biochemical characteristics that impart either activating or nonactivating properties to a material have not been completely described. However, current evidence supports the hypothesis that surface nucleophiles contribute importantly to the activating potential of a foreign material (FIG. 1). By constrast, negatively charged surface constituents facilitate factor H binding to C3b, thus causing a material to

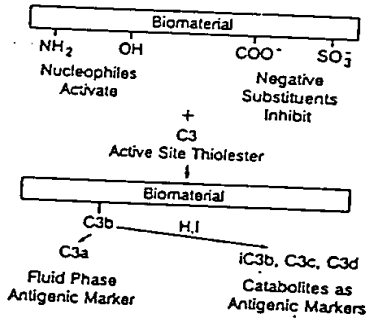

FIGURE 1. Schematic diagram summarizing the molecular mechanisms of the complement alternative pathway activation.

behave as a nonactivator. For example, the presence of sialic acid has been shown to promote high-affinity association between particle-bound C3b and factor H without influencing the affinity of C3b for factor B.[4] Additionally, chemical modification studies have clearly demonstrated that substitution of dextran with either carboxylic or benzylamine sulphonated groups conferred the capacity to inhibit formation of the amplification C3 convertase.[5] Finally, studies performed with particle-bound heparin provide an interesting example of the ability of anionic materials to act as inhibitors of the alternative pathway activation events.[6]

It is also important to note that factors H and I function as inhibitors of the alternative pathway because they are enzymes that cleave surface-bound C3b to yield the inactive catabolite iC3b (FIG. 1). Subsequently, other plasma enzymes such as plasmin kallikrein or elastase cleave iC3b to liberate the C3c fragment into the fluid phase while leaving the active site containing C3d fragment covalently attached to the surface. The final step in the breakdown and catabolism of C3 is hydrolysis of the ester or amide bond that covalently links the C3d fragment to the surface and resultant

liberation of this fragment from the material into the fluid phase. It should also be recognized that these same catabolic events take place when C3b becomes attached to an activating surface, although at a greatly diminished rate. Thus, activating biomaterials would be expected to display both surface-bound C3b and/or iC3b as well as measurable levels of C3c- and C3d-related antigens in the fluid phase after a reasonable period of incubation.

In summary, although both complement-activating and nonactivating materials may have surface nucleophiles, activating substances provide an environment that favors binding of factor B to C3b, thus facilitating feedback amplification of C3 convertase formation. As a result, an activating surface rapidly becomes saturated with C3b molecules and significant quantities of the C3a cleavage product are released into the fluid phase. By contrast, nonactivating materials frequently have negatively charged groups on their surface and are characterized by the fact that they promote binding of factor H to C3b. Consequently, nonactivating surfaces display a few bound iC3b molecules and levels of fluid-phase C3a antigen remain essentially unchanged. Subsequent catabolism of surface-bound C3b results in the time-dependent liberation of C3c, C3d, and other C3-derived fragments from the material surface into solution.

The Human Anaphylatoxins

The anaphylatoxins C3a and C5a are bioactive polypeptides that are produced during complement activation.[9] Human C3a is cleaved from C3 by the C3 convertase (C3b, Bb) that is formed during the amplification phase of the alternative pathway activation events. The C5 convertase C3b(n), Bb, formed by deposition of additional C3b molecules on the C3 convertase, acts to cleave C5 and liberate C5a into the fluid phase. Generation of these anaphylatoxin molecules by alternative pathway enzymes may be detected by employing specific radioimmunoassay (RIA) procedures.[10]

Additionally, RIA quantitation of both C3a and C5a antigens permits calculation of a constant designated the "coupling efficiency." This constant permits an assessment of the activating or nonactivating character of a material.[11,12] For example, potent activators of the alternative pathway, such as zymosan, behave as they do because they prohibit binding of factors H and I to C3b. Consequently, both the C3 and C5 convertases are readily formed on the particle surface and essentially equimolar (mole fraction) concentrations of C3a and C5a antigen are liberated into the fluid phase, *i.e.* the "coupling efficiency" of zymosan approaches 100%.[11] By contrast, factors H and I associate more readily with C3b when solution-phase activators, such as bacterial lipopolysaccharide, are employed as stimuli. Therefore, fewer C5 and C3 converting enzymes are established and the mole fraction of liberated C5a is less than that of C3a, *i.e.* the "coupling efficiency" of these weak activators is 25% or less.[11]

The anaphylatoxins that are produced when blood contacts a biomaterial are particularly important for at least two reasons. As depicted in FIGURE 2, the C3a antigen serves as a reliable plasma marker of complement activation because the [des-Arg-77] C3a ($C3a_{des\ Arg}$) polypeptide is biologically inert and accumulates readily in blood.[9] More importantly, the C5a and/or [des-Arg-74] C5a ($C5a_{des\ Arg}$) that is formed as a result of blood-material contact is a potent bioactive molecule that may provoke a variety of pathophysiologic responses.[9]

Extracorporeal production of C5a anaphylatoxin results in clinical manifestations that are directly related to the quantity of C5a produced at the blood-material interface. Generation of even modest amounts of this potent biological effector provides sufficient C5a to bind to the C5a receptor of peripheral blood granulocytes

passing through the extracorporeal circuit.[11] When this happens, the C5a-activated neutrophils and monocytes are returned to the patient, where they may become sequestered in the microvasculature. These biological events account for the granulocytopenia commonly observed during hemodialysis performed with complement-activating membranes. In addition to these acute phenomena, it has been suggested that C5a-stimulated neutrophil may initiate local tissue injury while it is in contact with the pulmonary endothelial cell and the C5a-activated monocyte might elaborate interleukin-1 (IL-1), which in turn could produce systemic manifestations of inflammation.[14]

It is also now apparent that C5a can have direct effects on the pulmonary vasculature. For example, both our published studies performed with swine[15] and our more recent unpublished investigations employing a sheep model demonstrate that intravenous infusion of either porcine or human C5a provokes a significant increase in the pulmonary artery pressure (PAP) and the systemic arterial pressure (SAP) while causing a dimunition in the cardiac output (CO) and hypoxemia. Interestingly,

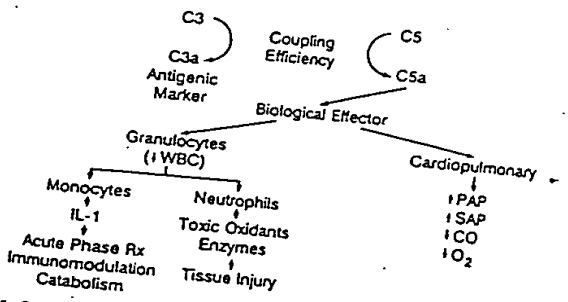

FIGURE 2. Schematic diagram depicting the biological consequences of anaphylatoxin production in extracorporeal circuits.

C5a-induced pulmonary hypertension is consistently detected prior to the onset of C5a-induced granulocytopenia, thus demonstrating the direct actions of the anaphylatoxin on the pulmonary vasculature.

COMPLEMENT ACTIVATION DURING HEMODIALYSIS

Observations

Initially, Craddock and his coworkers provided the first evidence of complement activation in extracorporeal circuits when they employed bioassays to demonstrate that C5a (des-Arg) resulted when blood contacted cellulosic hemodialyzer membranes. Subsequently, development of specific anaphylatoxin radioimmunoassays (RIAs) permitted extension of these observations to include evaluation of cardiopulmonary bypass circuitry and other types of hemodialyzers.[1] Currently, the greatest amount of

TABLE 1. Complement Activation and Granulocytopenia Produced by Various Dialyzers (in Normalized Percentage Values)

Dialyzer Type	Plasma C3a	Leukopenia
Cuprophan	100	100
Cellulose acetate	67	43
Hemophan	60	62
Polycarbonate	40	53
Polysulfone	24	12
Reused cuprophan	15	38
Polyacrylonitrile sodium methallylsulfonate (Hospal AN-69)	5	0

basic and clinical information has been obtained from studies of hemodialyzers. For this reason, it is worthwhile to review this data base and to attempt to correlate this information with what is known about the human complement system and the human anaphylatoxins.

The complement-activating potential of various types of dialyzer membrane materials can be clinically defined in two ways. First, the maximal amount of C3a antigen produced during the initial phases of hemodialysis can be quantitated by RIA methods. Second, the magnitude of the leukopenic response induced by hemodialysis with a particular type of dialyzer can be readily measured. As shown in TABLE 1, when the complement-activating potential of several different types of dialyzers is defined in this way, Cuprophan hollow fiber devices made with unmodified cellulosic membrane consistently produce the greatest amount of C3a generation and the greatest degree of leukopenia. Modification of the cellulosic material, either by acetylation (cellulose acetate) or introduction of tertiary amino groups (Hemophan), results in a 30–40% reduction in the maximum amount of plasma C3a detected during the first 15 minutes of dialysis. The leukopenic response provoked by these modified cellulosic dialyzers is also corresponding reduced. Perhaps the most "biocompatible" form of modified Cuprophan is that unique membrane which results from use and formalin storage of new dialyzers. Clinical studies performed with these reused Cuprophan dialyzers demonstreate that they produce only about 15% of the C3a formation induced by new dialyzers.[1]

Synthetic membranes consistently exhibit a lower complement-activating potential than cellulosic membranes. As shown in TABLE 2, both polycarbonate and polysulfone membrane dialyzers provoke only moderate C3a formation when evaluated clinically. The most interesting synthetic membrane appears to be a polyacrylonitrile-sodium methallylsulfonate copolymer, the Hospal AN-69 membrane. Dialyzers manufactured with this synthetic material produce only small quantities of C3a antigen that can be

TABLE 2. Classification of Hemodialyzers

Complement-activating Capacity	Biological Consequences	
	Granulocytopenia	Pulmonary Hypertension
high	yes	yes
moderate	yes	no
low	no	no

detected in the efferent (outlet) line of the dialyzer and fail to induce granulocytopenia in the dialyzed patient.[2]

In addition to direct quantitation of C3a antigen and determination of peripheral blood leukocyte counts, the complement-activating potential of dialyzers may be defined by physiologic measurements. For example, as mentioned previously, purified porcine C5a produces a significant increase in the pulmonary artery pressure when injected intravenously into swine.[4] Similar results have been observed when sheep were employed as the test animal (unpublished observation). Thus, measurements of PAP during dialysis performed on swine or sheep have been exploited to estimate the amount of C5a produced during the procedure. These types of studies have demonstrated that unmodified cellulosic membranes produce a two-to-threefold elevation of the mean PAP within 5 minutes after the onset of dialysis. By contrast, the newer modified cellulosic membranes (Hemophan), although they still activate complement with 60% of the activity of Cuprophan, do not provoke this physiologic response. Similarly, the synthetic membranes that display reduced complement-activating potential, such as polysulfone and AN-69, also fail to produce a significant increase in PAP during dialysis.

TABLE 3. Factors Governing Patient Exposure to C5a Anaphylatoxin during Extracorporeal Circulation

I. Factors effecting complement activation
A. Number of reactive groups
B. Reactivity of surface groups
C. Nature and number of surface regulatory constituents
II. Factors effecting anaphylaxtoxin distribution
A. Transport through materials (membrane)
B. Binding to materials
C. Interaction with blood cells in the extracorporeal circuit

In summary, detailed clinical and laboratory studies suggest that hemodialyzer membrane materials tend to fall into one of three different categories (TABLE 2). The first category is exemplified by unmodified cellulosic membranes. These display a great capacity to activate complement and are characterized by the fact that they provoke both granulocytopenia and pulmonary hypertension. The second group consists of a large number of modified cellulosic and synthetic membranes that display about half the complement-activating potential of Cuprophan. These membranes are distinctly different from unmodified cellulosic ones because, although they do induce pulmonary vascular leukosequestration, they fail to promote pulmonary hypertension. The Hospal An-69 membrane is most typical of the final category. Dialyzers containing this synthetic copolymer display low complement-activating potential and fail to induce either granulocytopenia or pulmonary hypertension.

Hypotheses

Results of studies performed to date suggest that there is one central issue to resolve when considering complement activation in extracorporeal circuits. That is to identify the factors that govern the quantity of the bioactive C5a molecule is delivered from the extracorporeal circuit to the patient during the therapeutic procedure.

Knoweldge of the mechanisms of complement activation, the biological properties of human C5a anaphylatoxin, and the observed performance of different hemodialysis membrane materials permits a hypothetical delineation of these critical factors and provides a conceptual framework for understanding biomaterial-complement interactions.

The author speculates that the factors that account for patient exposure to C5a anaphylatoxin during extracorporeal circulation can be divided into two broad categories. First, it is postulated that certain membrane characteristics will effect the complement activation process directly (TABLE 3). For example, experimental evidence supports the contention that both the relative number and chemical reactivity of surface nucleophiles contributes significantly in this regard.[13]

Additionally, it is now appreciated that other membrane surface groups may play a critical role in regulating surface activation events. Particular emphasis is being placed on those that are negatively charged. These regulatory phenomena may be experimentally manifested as a reduction in the "coupling efficiency."

Second, factors that effect anaphylatoxin distribution would also be expected to modify patient exposure to C5a that is produced within the extracorporeal circuit. In this case, emphasis has been placed on the fact that the low molecular weight cationic C5a molecule may either by transported through certain porous membranes or bound tightly to other anionic membranes. For example, the apparent "biocompatibility" of the AN-69 membrane appears to result from the fact that it binds both C3a and C5a after they are produced.[14] Finally, basic investigations have demonstrated that C5a is irreversibly bound to granulocytes under physiologic conditions. Thus it is likely that binding of this anaphylatoxin to the cellular elements of blood in the extracorporeal circuit induces cellular activation while reducing the plasma levels of the glycopolypeptide. As a consequence, patients may exhibit granulocytopenia but be spared from exposure to the freely circulating bioactive C5a.

SUMMARY

Clinical studies performed with various types of hemodialyzers provide a data base and conceptual framework for understanding blood-material interactions that result in complement activation. Current findings suggest that the complement-activating potential of biomaterials may be defined by quantitating either fluid-phase C3a antigen or measurement of surface-bound C3 fragments. Furthermore, systematic studies can reveal material properties that regulate both the production and distribution of the human anaphylatoxins. Assessing the fate of the C5a anaphylatoxin is particularly important because it is likely that generation of this bioactive molecule in extracorporeal circuits initially triggers granulocyte responses and may produce cardiopulmonary manifestations if the C5a is formed in sufficient quantities. It seems likely that detailed knowledge of these varied phenomena will permit rational design of newer "biocompatible" materials and membranes.

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CHAPTER 11
Pharmacologic Modification of Materials

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Several approaches have been used to develop biomaterials with pharmacologic activity for use in cardiovascular medicine. These approaches depend upon whether the active agent is to be: (i) released slowly from the device; or (ii) retained in bound form indefinitely. The approaches also depend upon whether the pharmacologic activity of the agent is designed to modulate local: (i) coagulation; (ii) platelet function; or (iii) cell function in the vessel.

Bound versus Releasable Agents

The selection of a bound versus a releasable agent depends upon the desired duration of activity and the mode of action of the agent. Agents that are active intracellularly, that must be internalized for activity, or that exert their activity by binding to the luminal surfaces of the attached cells are presumably inappropriate for immobilization for indefinite retention. An example of such an agent would be polypeptide growth factors, which bind to cell-surface receptors; the receptors are subsequently clustered to promote activity and are ultimately internalized. Agents that are active extracellularly may be indefinitely retained or may be released, depending upon the desired duration and location of response. An example of such an agent would be heparin, which accelerates the inactivating reaction of antithrombin III (AT III) with thrombin to prevent coagulation.

Antithrombin, Antifibrin, or Antiplatelet Agents

Pharmacologic approaches to reduce thrombosis can be directed against the formation of fibrin or against the platelet. Antifibrin agents include thrombin inhibitors and fibrinolytic agents or modulators of fibrinolytic activity. The most common thrombin inhibitor is heparin, and heparin incorporation has been examined extensively. Another thrombin inhibitor that will likely receive more attention is hirudin. Agents participating in fibrinolysis are plasmin and activators of plasminogen, such as streptokinase, urokinase, and tissue plasminogen activator. Antiplatelet agents include prostacyclin, other prostaglandins, and receptor antagonists, such as bioactive peptides that competitively inhibit the interaction between glycoprotein IIb/IIIa and fibrinogen, as well as antibodies directed against glycoprotein IIb/IIIa or glycoprotein Ib. Aspirin would likely not be considered due to the ease and economy of systemic treatment without significant side effects.

Promotion of Cellular Response: Endothelial/Neointimal Adhesion and Growth

An approach to biocompatibility in cardiovascular materials is to promote the rapid coverage of the biomaterial by cells from the vessel wall, namely endothelial cells and perhaps also vascular smooth muscle cells. The biocompatibility of the composite would then depend largely upon the function of the adherent cells and more indirectly upon the biomaterial and its ability to promote endothelial cell attachment and to support the normal antithrombotic function of the cells. An approach to pharmacologic activity or bioactivity is then to incorporate agents that promote this response, such as species that mediate cell adhesion or cell growth. For example, fibronectin and laminin, or their active receptor-binding domain peptides, may be immobilized on

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a surface to promote endothelial cell adhesion; in this case the agent must presumably remain surface-bound for long periods of time. Polypeptide growth factors that have activity toward vascular endothelial cells may be incorporated to promote growth rather than adhesion; the most important such factors are probably acidic and basic fibroblast growth factor. In this case, the agent should most likely be incorporated in a releasable manner.

Surfaces Modified with Heparin

Without question the most extensively studied pharmacologic modification of cardiovascular biomaterial surfaces is the incorporation of heparin to obtain local anticoagulant action. Heparin has been incorporated using chemistries for both release and long-term retention at the surface (1).

Mode of Action of Heparin

Heparin is a natural polysaccharide consisting of modified saccharide repeats based upon uronic and glucosamine linked by β-D and α-D 1-4 bonds (2). Heparin is a heterogeneous substance, with molecular weight distribution being the most important parameter; the molecular weight of commercial heparins may vary from 3,000 to 30,000 daltons. Heparin interacts with plasma AT III to form a heparin:AT III complex that inactivates thrombin by forming the inactive heparin:AT III:thrombin (HAT) complex. Thus, heparin is a cofactor that accelerates the inactivation of thrombin by AT III. Only about one-third of the molecules in standard commercial heparin exhibit high-affinity binding with AT III.

Heparin is synthesized in the form of a proteoglycan, the carbohydrate portions of which are subsequently released by proteases or glycosidases (3). The uronic and glucosamine residues are highly modified by sulfation, deacetylation, and epimerization. The structure may be represented by a hexasaccharide repeat, as illustrated in Figure 1, but the polymer should be understood to be much more heterogeneous than is shown.

Chemistry of Heparin Incorporation

Heparin has been incorporated into biomaterial surfaces for slow release and for prolonged retention by a very large number of chemical schemes. The functional groups available on heparin for modification include hydroxyls, carboxyls, and primary amines. A number of examples are presented below, but this collection of examples should not be considered complete.

Heparin has been bound to materials for slow release by ionic interaction. To accomplish this, the material surface is somehow rendered cationic, to lead to strong adsorption of the overall anionic heparin polymer. One approach for achieving this surface modification involves the absorption of bifunctional agents, one function of which is cationic, such as a quaternary amine, and the other function of which is hydrophobic. Examples of such agents are benzalkonium chloride (3) and tridodecylmethyl ammonium chloride (4). The quaternary amines are all readily prepared by quaternizing an amine, such as a primary amine or a pyridine moiety as a starting agent, with a bromoalkane: for example, quaternizing pyridine with cetylbromide to form cetylpyridinium bromide. Since the heparin is merely adsorbed, it slowly leaches from the surface under physiologic conditions, and the kinetics of release depend upon the extent of surface treatment with the cationic agent. Estimates of the rate of leaching required to obtain biological activity have been determined mathematically (5). As an alternative to this approach, the heparin may be directly adsorbed to a polymer that bears cationic functions at its surface, such as polyethylene imine (6); in particular, polymers bearing amido amine moieties bind heparin strongly (7).

A second approach to obtaining slow release of heparin is to incorporate it by blending it with the polymer of interest. In this case, release can occur by leaching or by degradation of the base material, for example, by utilizing degradable materials of poly(α-hydroxy acids) (8). To obtain release at appreciable rates in the absence of degradation, polymers must be used that have relatively high degrees of chain mobility, such as silicone rubber or hydrogels (9).

Many thermochemical approaches have been used to graft heparin to polymers. These include activation of surface nucleophiles with glutaraldehyde, leaving one aldehyde for reaction with heparin hydroxyls and amines (10), and activation of surface nucleophiles for subsequent reaction with heparin hydroxyls and amines, using coupling agents such as cyanogen bromide (11), carbodiimide (12), bifunctional isocyanates (13,14), or bifunctional isocyanates grafted to polyamines grafted to the substrate (15).

The conformation of the incorporated heparin may be important in determining its biological activity. This is due to the requirement that it form a complex with both thrombin and AT III. Heparin immobilization by chemical modification of a terminal saccharide residue, to achieve end-grafting, has been demonstrated to enhance activity (10,16,17). The issue of accessibility has been addressed by immobilizing the heparin via a hydrophilic spacer arm, such as polyethylene glycol (16–18). Polyethylene glycol spacers from 2,000 to 4,000 g/mol appear to be optimal for preservation of the activity of the immobilized heparin, even to the extent of increasing activity by a factor of 10 (19). This increase in activity is likely due to increased steric availability to bind with AT III and heparin. The immobilization in vivo has led to considerable prolongation of patency of vascular grafts in dogs (20).

One particular form of heparin immobilization utilizing end-point attachment of heparin fragments has been commercialized by Carmeda AB and several corporate partners (21, 22). Heparin is cleaved with nitrous acid, producing a terminal aldehyde. This reactive heparin molecule is then coupled to a primary amine on a surface, usually from polyethyleneimine. To obtain high densities of amine, alternating layers of anionic polysaccharides, such as chon-

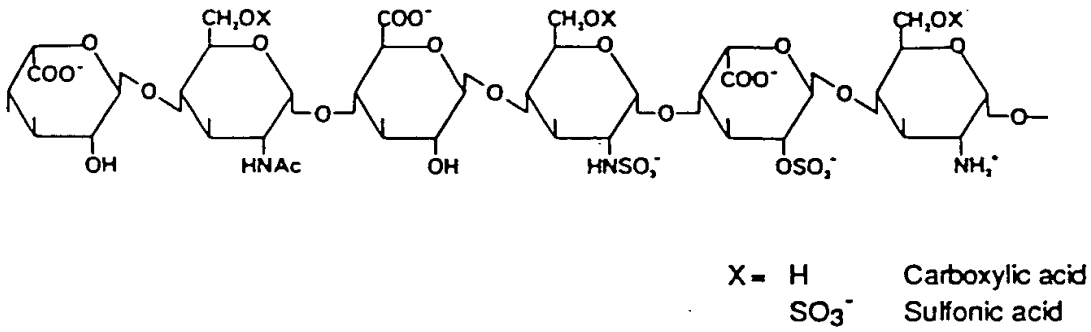

Figure 1. Generalized chemical structure of heparin at physiologic pH. Chemical moieties available for coupling are hydroxyls, amines, carboxylates, and sulfates.

droitin sulfate, and polyethyleneimine are sometimes used, with heparin-aldehyde coupling to the outermost layer. The resulting Schiff base is then reduced to a secondary amine using, for example, sodium cyanoborohydride.

Behavior of Incorporated Heparin

The amount of heparin incorporated may be determined by several means standard to polymer grafting. Such means include x-ray photoelectron spectroscopy and the use of radiolabeled heparins, the latter of which is more quantitative. Staining techniques are also useful; toluidine blue reacts readily with heparin, producing a metachromatic shift (10). This shift may be calibrated with known amounts of heparin to determine, at least semiquantitatively, the amount of heparin bound and/or released. Alcian blue has been used to detect as little as 10 ng of immobilized heparin (23). Measurement errors may be associated with this technique, however, if the immobilization chemistry significantly alters the chemical structure of the heparin or if it selects for particular fractions of heparin, in which cases free or heterogeneous heparin may not serve as an appropriate calibration control.

The biological activity of the surface-bound and/or released heparin may be tested by several means. Biological assays, such as measurement of the thrombin time or whole blood clotting time, may be useful, but the direct measurement of the ability of the bound and/or released heparin to inactivate thrombin or to catalyze the formation of the thrombin:AT III complex probably yields more insight and a clearer interpretation. The activity of thrombin may be readily determined by a chromogenic substrate assay (for thrombin) using the peptide S-2238 (H-D-Phe-Pip-Arg-NH-benzyl-NO_2 · HCl, KabiVitrum). The formation of the complex may be determined by use of radioimmunoassays or enzyme-linked immunosorbent assays for the thrombin:AT III complex or for the heparin:AT III:thrombin complex. Features that are of particular interest in such determinations are the specific activity of the immobilized heparin (i.e., the ability to catalyze the formation of the HAT complex per mole of heparin, relative to that of a free heparin standard), the rate of leaching, whether intentional or not, and whether the immobilized heparin is catalytic or is consumed. The stability and release characteristics must be determined over a period of many days to examine leaching. The activity of the immobilized heparin should be measured upon a surface not previously exposed to thrombin and AT III, upon a surface previously exposed to thrombin and AT III, and on a surface previously exposed to blood. It should also be noted that some heparin preparations may cause platelet aggregation; accordingly, platelet interactions with the surface-bound heparin should be examined. The required release rate and desired coating lifetimes determine the amount of heparin to be incorporated. Alternatively viewed, the actual release kinetics and amount of heparin incorporated determine the useful lifetime of the coating.

A critical issue in immobilized heparin intended for long-term retention is whether it functions in a catalytic manner when immobilized, i.e., whether it is capable of accelerating the inactivation of thrombin by AT III after repeated exposure to thrombin and AT III. For example, essentially complete retention of activity has been observed through many exposure cycles in vitro (11), and the ability to bind thrombin with high affinity has been retained after periods of implantation of up to 4 months (24). Increased patency was observed in a canine, short-term ex vivo arteriovenous shunt when heparin was immobilized to the surface compared with controls (25), although efficacy in chronically shunted animals was limited due to platelet consumption (26) that was likely due to the base material. Prolongation of patency, by a factor of 2.5, in 1 mm diameter by 1 m long arteriovenous shunts in the dog containing end-grafted heparin upon polyethylene and polyurethane tubes has been observed (27).

Care should be taken when interpreting biological behavior on contact with whole blood, for example, in measurements of platelet attachment or whole blood clotting time. Multiple factors are altered by the attachment of heparin to a substrate, namely the physicochemical nature of the surface, which may result in a change in biological behavior independent of the anticoagulant activity of heparin as well as the anticoagulant activity of the surface. It

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is not possible to decouple these two changes, but it is sometimes illustrative to perform parallel experiments with polysaccharides that have lesser anticoagulant activity, such as dermatan sulfate.

Surface Modification with Other Anticoagulant or Antiplatelet Agents

Hirudin

Hirudin was originally found in medicinal leeches (28,29), *Hirudo medicinalis*. The protein is a 65 amino acid residue monomer with a molecular weight of 7,000 daltons, an isoelectric point of 3.9, and no carbohydrate content. There are several forms of hirudin from leeches that differ slightly from each other in primary structure. They are known as HV1, HV2, HV3, and PA-hirudin. Recombinant hirudin HV1 has been produced in *Escherichia coli* and differs from leech HV1 only in the state of sulfation of the tyrosine residue at position 63: the leech HV1 Tyr 63 is sulfated, while the *E. coli* HV1 is not. This small difference results in about a tenfold difference in the bioactivity of recombinant hirudin compared with hirudin derived from leeches.

Hirudin binds very specifically and with high affinity to thrombin, and the hirudin:thrombin complex lacks coagulant activity (30). Hirudin lacks the potential to aggregate platelets, in contrast to some fractions of heparin (31). Because the binding to thrombin is of such high affinity, hirudin that is indefinitely bound to a surface may be ineffective in reducing the potential of a material to promote coagulation over a long period, since the hirudin may be permanently occupied by the thrombin to which it first binds. This may limit the effective mode of hirudin incorporation into surfaces to that of controlled release.

The covalent immobilization of hirudin to surface-hydrolyzed Dacron by carbodiimide-grafting chemistry with the retention of antithrombin activity has been described (32), and the prolongation of plasma thrombin clotting times in the presence of immobilized hirudin has been reported (33).

Plasmin and Plasminogen Activators

Fibrinolysis (34), i.e., the degradation and dissolution of fibrin, is mediated by plasmin, which in turn is generated by the proteolysis of plasminogen under the action of tissue-type plasminogen activator (tPA) or urokinase (see Chapter 3). These agents represent potential targets for controlled release from biomaterial surfaces, although relatively little work has been done in this area. It seems unlikely that indefinitely retained plasmin will be useful, since the substrate fibrin is essentially a solid and the approach of the solid substrate to immobilized enzyme may be sterically difficult. The indefinite retention of tPA or plasminogen may, however, prove useful, since the substrate for these enzymes, plasminogen, circulates in solution. A system has been developed for the release of immobilized streptokinase (a streptococcal activator of fibrinolysis) within a poly(methacrylic acid-g-ethylene oxide) copolymer; a high degree of retention of enzymatic activity was observed (35). Immobilized streptokinase has been examined in vivo (36), and retention of fibrinolytic activity was observed for greater than 150 days. The kinetics of immobilized urokinase has been examined in vitro (37).

Antiplatelet Agents

A variety of antiplatelet agents are candidates for incorporation into biomaterials for controlled release (38), although relatively little work has been done in the area. Prostaglandins have been incorporated into materials for both release and retention. The prostaglandins PGI_2, PGE_1 and PGD_2 all stimulate adenylate cyclase to increase intracellular cAMP (in order of decreasing potency), which in turn reduces the potential of the platelet to become activated. PGE_1 released from a polyurethane in contact with rabbit blood ex vivo (39) was shown to reduce platelet activation, and PGE_1 retained on the surface of Sepharose was shown to increase platelet adhesion but to decrease the level of platelet activation (40).

Antibacterial Agents

Antibacterial agents, both antibiotics and less specific agents, have been incorporated into cardiovascular materials for controlled release. Dicloxacillin has been incorporated into catheter formulations by coating with an antibiotic-quaternary amine complex (in a fashion similar to the incorporation of heparin, as described above) and by combining with a polyurethane matrix (41); these materials release inhibitory doses for at least 24 hours. Other antibiotic coatings have been developed for a silver-tolbutamide complex (42) and a silver-ciprofloxacin complex (43), the latter of which was observed to release over a 14-day period. Antibiotic binding to quaternary amines has been investigated and used in the limitation of infection in Dacron vascular graft prostheses (44).

Surfaces Modified with Proteins or Peptides for the Promotion of Endothelialization

Approaches to increase the extent and rate of endothelial cell attachment to cardiovascular materials have included such pharmacologic approaches as the preadsorption of cell adhesion proteins, including collagen, fibronectin, vitronectin, and laminin. One difficulty in this approach is that an increase in endothelial cell adhesion can be accompanied by an increase in platelet adhesion and subsequent thrombosis. Newer approaches, as yet untried in vivo, have used covalently attached cell adhesion peptides, derived from the domains of the cell adhesion proteins listed above that are involved in receptor binding, to enhance endothelial cell adhesion in a more selective fashion.

Endothelialization of vascular grafts may play an important role not only in the reduction of thrombosis but also in the control of neointimal thickening and anastomotic

hyperplasia (45,46). In an organ culture model, smooth muscle cell proliferation was reduced in vessels that were endothelialized compared with those that were not endothelialized (45), suggesting the release of a smooth muscle cell regulating factor by the endothelial cells; these same control mechanisms may be important in the control of hyperplasia, or the lack thereof, in vascular grafts. Endothelial cells may arise by migration through the porous wall of the graft (47), or they may be placed by seeding or sodding (48).

Collagen, Fibronectin, Vitronectin, Laminin, and Peptides and Their Mode of Action

Collagen, fibronectin, vitronectin, and laminin are all cell adhesion proteins for which cell-surface adhesion receptors on the endothelial cell exist (49). Many of these receptors are members of the integrin superfamily of receptors and consist of heterodimers of an α and a β subunit. Endothelial cells (50,51) also express the β_1 group integrins $\alpha_v\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$. These integrins promote adhesion to collagens ($\alpha_3\beta_1$), laminin ($\alpha_2\beta_1$, $\alpha_6\beta_1$), and fibronectin ($\alpha_5\beta_1$). Endothelial cells contain the β_3 group integrin $\alpha_v\beta_3$, which promotes adhesion to vitronectin, von Willebrand factor, fibrinogen, thrombospondin, and fibronectin. This topic is covered in greater detail in Chapter 14.

The domains of cell adhesion proteins that bind to their corresponding receptors have been described. The prototypical motif for the domains that bind to the integrin family of receptors is based on the tripeptide sequence Arg-Gly-Asp (RGD) (52); the sequences in collagens are Arg-Gly-Asp-Thr and Asp-Gly-Glu-Ala; the sequences in fibronectin are Arg-Gly-Asp-Ser, Leu-Asp-Val, and Arg-Glu-Asp-Val; the sequence in vitronectin is Arg-Gly-Asp-Val; and the sequences in laminin are Leu-Arg-Gly-Asp-Asn and Tyr-Ile-Gly-Ser-Arg (for which a non-integrin receptor exists), among others. These sequences, which may be readily synthesized, bind to their receptors with high affinity, albeit with less affinity than that of the intact protein.

Chemistry for Incorporation

The goal of the immobilization chemistry is to retain the immobilized agents for long periods in bioactive form. The most common technique is simple adsorption, and this has indeed been shown to increase the rate and extent of endothelial attachment and spreading. Difficulties with adsorption are the potential for exchange and the susceptibility of the adsorbed protein to denaturation due to interaction with the surface or to proteolysis by the cell. Nevertheless, the pretreatment of cardiovascular surfaces with adsorbed cell adhesion proteins, for example, fibronectin or laminin, can dramatically increase the rate of spreading and the strength of binding of seeded endothelial cells, as outlined further, below. Due to the instability of the proteins in vivo, under the proteolytic action of the cells, no advantage is apparent when coupling the proteins by covalent means rather than adsorption.

Synthetic RGD-containing peptides have been immobilized by both adsorption and covalent binding. An RGD-containing peptide with a hydrophobic oligoleucine tail has been developed for adsorption to hydrophobic surfaces (53). RGD-like peptides have been covalently immobilized to a variety of surfaces using standard protein immobilization chemistry (54,55).

Behavior of Incorporated Agents

The pretreatment of cardiovascular material surfaces with proteins such as fibronectin has been shown to enhance both the rate and extent of endothelial cell spreading in vitro in numerous investigations (for example, see references 56 and 57). More significantly, pretreatment of expanded polytetrafluoroethylene (ePTFE) grafts with fibronectin was demonstrated to enhance endothelialization of the graft in vivo, albeit in dogs (58).

Surfaces bearing covalently immobilized RGD-like peptides were shown to support morphologically complete cell adhesion and spreading in vitro, even in the absence of potentially adsorbing adhesion proteins (54,55). One difficulty in the use of adsorbed proteins or peptides to promote endothelial cell adhesion is that thrombosis may also be promoted inadvertently. To address this problem, the fibronectin peptide Arg-Glu-Asp-Val was immobilized on otherwise poorly adhesive substrates and shown to have affinity for endothelial cells but not for vascular smooth muscle cells, fibroblasts, or blood platelets (59), suggesting the potential for a graft material that is selective for endothelial cells. Important issues in these approaches will be the stability in vivo and competition or masking by adsorbing proteins. Stability for periods of at least several months is possible if protease-sensitive sequences are avoided, or if D- to L-amino acid substitutions are used where possible. Masking by potentially adsorbing proteins has not been observed in vitro in serum-containing media (54,55,59), but behavior in vivo awaits determination.

Surfaces Modified with Growth Factors for the Promotion of Endothelialization

Polypeptide growth factors, particularly basic fibroblast growth factor and endothelial cell growth factor, have been determined to play a role in angiogenesis (60). Relatively few studies have been reported on the incorporation of such factors into the walls of vascular grafts for slow release in vivo, although it will likely become an important area of investigation. One example is an incorporation and release scheme for endothelial cell growth factor that permits its liberation from a treated Dacron graft for periods of several days in vivo (61).

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EMERGING TECHNOLOGIES

Jan C. Horrow, MD, Section Editor

Heparin-Coated Cardiopulmonary Bypass Circuits

Glenn P. Gravlee, MD

WHEN experimentation with extracorporeal circulation began in the late 1800s, one of the first problems encountered was the need to prevent clots from forming when blood contacted foreign surfaces. The idea of using heparin for this purpose has been attributed to Gibbon (A. Keats, personal communication, January 1993), who also performed the first successful surgical procedure in humans using extracorporeal circulation and gas exchange.[1] Heparin has served as the mainstay of anticoagulation for cardiopulmonary bypass (CPB) for almost four decades because it has a rapid onset, it effectively prevents clot formation, and it is rapidly and completely neutralized by protamine. Since the clinical introduction of CPB in the 1950s, probably anticoagulation and its neutralization have changed less than any other aspect of CPB management. The only major advance in anticoagulation has been the use of monitoring to achieve more consistent endpoints for anticoagulation and its reversal.[2,3] Some have questioned whether even this constitutes an improvement over empiric dosing of heparin and protamine.[4]

Though it might be tempting to attribute the longevity of heparin and protamine to their superior pharmacologic properties, these drugs possess some important limitations. Heparin activates and degranulates platelets,[5] which may contribute to the well-documented platelet functional deficit that follows CPB. In the absence of heparin or other anticoagulants, fibrinogen rapidly coats foreign surfaces, and platelets adhere to fibrinogen soon thereafter.[6-8] Systemic administration of heparin, even in the enormous doses used for CPB, incompletely prevents the adherence of fibrinogen and platelets to synthetic surfaces.[9] Blood coagulation remains moderately active during CPB despite profound anticoagulation with heparin.[10,11] Protamine activates complement, causes vasodilation, and can cause life-threatening anaphylaxis or pulmonary hypertension.[12] Optimal protamine dosing remains controversial, because inadequate doses fail to neutralize heparin-induced anticoagulation while excessive doses probably exacerbate coagulopathy after CPB and may contribute to adverse hemodynamic side effects of protamine.[13-15] It appears that the use of heparin and protamine for CPB continues at least in part because no practical alternatives have been introduced.

In the vasculature, several systems work in concert to maintain the fluidity of the bloodstream. Endothelial cells secrete the platelet inhibitor prostacyclin and the endothelial surface intrinsically inhibits thrombin, which is a potent stimulant of both platelet aggregation and fibrin formation. A heparin-like glycosaminoglycan called heparan sulfate adheres to the endothelial surface and attracts circulating antithrombin III, which largely accounts for the thrombin inhibition.[16] It has long been thought that a nonthrombogenic synthetic surface would greatly enhance the biologic compatibility of extracorporeal circulation. In theory, the more closely this surface reproduced the normal interface between blood and endothelium, the more compatible it would be.

HISTORY

In 1963, Vincent Gott, a prominent researcher and cardiothoracic surgeon, reported a technique for coating plastic surfaces with heparin via its attachment to a colloidal graphite coating.[17] Some found this surface impractical because of the opacity and brittleness of the graphite coating.[18] Soon after Gott's discovery, others achieved heparin binding by using quaternary ammonium salts to link heparin to synthetic surfaces, which usually consisted of polyethylene or polyvinylchloride.[18-22] This technique has been applied to extravascular shunts, intravascular catheters, and to extracorporeal oxygenator circuits. It has commonly been termed "ionic heparin binding," which takes advantage of heparin's polyanionic nature to bind it to a positively charged quaternary ammonium ion, which binds in turn to the synthetic surface. This is similar to the molecular interaction between heparin and protamine, except that each protamine molecule binds a heparin molecule at multiple sites, thus covering the attachment sites for antithrombin III. Surface attachment of heparin would more likely bind each heparin molecule in one or two places, thus leaving exposed the critical pentasaccharide sequence that binds antithrombin III, and possibly sites for binding heparin cofactor II as well.[23]

In the 1970s various methods for binding heparin to ammonium ions were used to coat the surfaces of extravas-

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cular shunts and of extracorporeal membrane oxygenators for venovenous bypass in experimental animals.[20,24-27] Some results were favorable over periods ranging from 1 to 5 days without systemic heparin administration.[20,26,27] One report showed reduced survival with this technique as compared to 24-hour perfusions with traditional systemic heparinization or to perfusion without systemic heparin or a heparin-coated bypass circuit.[25] Another group showed promising results in both animals and humans using venoarterial bypass with a heparin-free nonthrombogenic surface composed of polyurethane-polyvinylgraphite.[28-30] One problem with some of these binding techniques was that the heparin would gradually leach off of the surface, leaving the ammonium-containing ligand exposed. This substrate might attract and bind platelets or be released into the bloodstream to cause potential toxicity.[31]

In patients requiring long-term extracorporeal oxygenation for pulmonary support, avoidance of systemic anticoagulation seemed ideal because hemorrhagic complications frequently ensued in that setting. Ionic binding of heparin to oxygenator surfaces presented other problems, however, such as the tendency of certain surfaces to swell and occlude, especially at sites of connection.[20] This necessitated the use of a different molecular source of ammonium ion for different sites within the circuit, thus precluding the desirable possibility of assembling an entire disposable circuit and flushing it with a single solution that would render it receptive to heparin coating. Because heparin washout occurred over time,[20] the duration of protection induced by this type of heparin coating was difficult to ascertain. Before these problems could be resolved, the clinical use of extracorporeal oxygenation for pulmonary support lost favor as a result of poor clinical outcomes.[32] Possibly because of a less urgent need to reduce or eliminate systemic anticoagulation for the much shorter periods of extracorporeal circulation used for cardiac surgical procedures, subsequent technologic advances in heparin-coated extracorporeal surfaces did not reach clinical trials until recently.

TECHNIQUES OF HEPARIN COATING

Figure 1 schematically demonstrates the most common techniques for attaching heparin to a synthetic surface, which were recently reviewed by Hsu.[33] Heparin, a complex macromolecular acidic organic polysaccharide, is highly negatively charged at physiologic pH. Heparin's hydrophilic nature complicates its attachment to biomedical polymers, most of which are hydrophobic.[31] Even within a particular category such as ionic or covalent heparin binding, specific molecular links vary in their affinity for heparin. Consequently, the duration of heparin attachment during continuous perfusion varies, as does protection against thrombosis at the interface between the blood stream and the synthetic surface. In general, covalent linkages appear more stable than ionic ones,[34] and less thrombogenic.[23] However, several techniques exist for covalent and ionic heparin binding, so the merits of covalent versus ionic binding techniques cannot be judged generically. Other considerations include the availability of attachment sites for antithrombin III and the possibility of heparin metabolism by circulating enzymes. Some configurations might limit exposure to circulating antithrombin III,[35] which is probably the most important mechanism of protection against thrombosis upon non–heparin-coated surfaces.

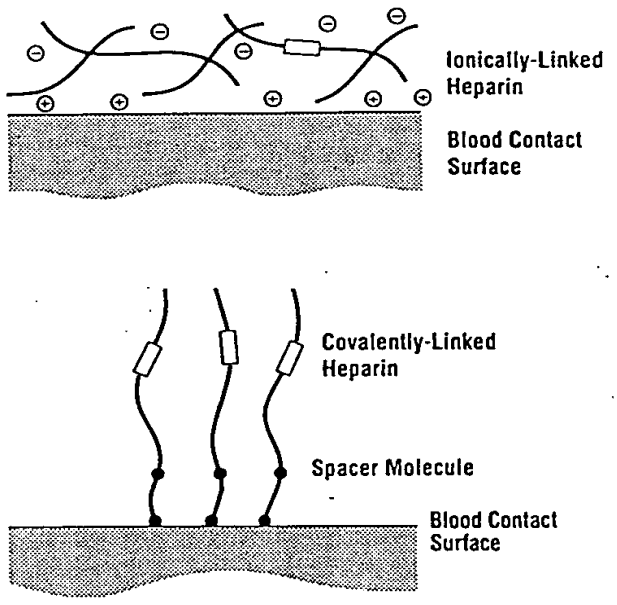

Fig 1. Schematic representation of ionic and covalent methods for binding heparin to a prosthetic surface. Spacer molecules (wavy lines between closed circles) as shown in the covalently linked heparin diagram are also commonly used in ionic linkages. Rectangles represent antithrombin III binding sites. (Courtesy of Medtronic Cardiopulmonary, Anaheim, CA.)

Even if thrombin is avidly bound by a heparin-coated surface, antithrombosis appears to depend upon the simultaneous interaction of thrombin, antithrombin III, and heparin.[35] Although activated factor X (Xa) contributes importantly to physiologic thrombogenesis, successful inhibition of thrombin appears more important to an antithrombogenic interface between whole blood and a foreign surface than inhibition of Xa.[36] Much like the physiologic interaction among heparan sulfate, antithrombin III, and thrombin at endothelial surfaces, the extracorporeal interaction among heparin, antithrombin III, and thrombin is a regenerative one.[37] This means that the heparin-antithrombin III-thrombin surface complex releases stable thrombin-antithrombin III complexes into the bloodstream, thus freeing the surface-bound heparin to interact repeatedly with circulating unbound antithrombin III and thrombin.

Other important considerations include the flow rate in the extracorporeal circuit and the potential for blood stasis. Blood tends not to clot if it is flowing quickly through an extracorporeal conduit, even if the conduit material is intrinsically thrombogenic.[38] Conversely, blood will tend to form clots when it flows slowly through a synthetic conduit even if the conduit is heparin-coated.[26,39] Even if flow is brisk, the complex components and connections in extracorporeal circuits used for cardiopulmonary bypass tend to create areas of stasis.

Spacer molecules interposed between the synthetic sur-

face and heparin increase heparin interaction with antithrombin III.[34,37,40-42] The length and the chemical composition of the spacers influence both the binding stability and the antithrombotic efficacy of the surface-bound heparin. Chains composed of polyethylene oxide appear more efficacious than those of alkane composition.[41] In general, longer spacer chains appear more antithrombotic than shorter ones.[37,41] Some have proposed that longer spacer chains provide more molecular mobility, greater bulk, more exposed antithrombin III binding sites, or the potential for heparin molecules to bind to the spacer molecules.[34,41] Glutaraldehyde treatment of the heparin-treated surface also appears helpful.[37]

Platelet interaction represents another important aspect of surface-bound heparin. Although thrombogenicity and platelet deposition are often engendered by the same stimuli, these two processes are not always interdependent. Commercial heparin preparations contain a wide range of molecular chain lengths (approximately 5,000 to 50,000 D), and longer heparin chains strongly bind and activate platelets, causing them to degranulate.[37] This characteristic of heparin would therefore tend to compromise antithrombogenesis at the interface between blood and a heparin-coated artificial surface. Heparin's propensity to bind platelets can be opposed in at least two ways: (1) reducing the mean molecular weight of the surface-bound heparin, or (2) interposing platelet-repelling proteins between the heparin-coated surface and circulating platelets.[37] Both techniques have been used effectively.[37] Even in the presence of a heparin-coated surface, plasma proteins tend to deposit at the blood-surface interface before platelets do. This deposition shields platelets from heparin, particularly if the protein is albumin.[40,43] The interactions at a heparin-coated surface are demonstrated schematically in Fig 2. Even fibrinogen coating can protect against platelet binding and activation as long as the heparin-coated surface prevents thrombin-mediated conversion of fibrinogen to fibrin. Protein denaturation facilitates platelet adhesion and activation.[43,44] The nature of spacer molecules used to connect heparin to the synthetic surface also influences platelet binding, although the length of the spacer molecule appears to have no effect.[40,41]

Two currently available products permit extracorporeal circulation using heparin-bonded surfaces. One technique deposits a polyethylenimine (PEI) spacer onto the various extracorporeal surfaces, then attaches heparin fragments created by nitrous acid degradation of commercially available porcine mucosal heparin.[23,33] Nitrous acid degradation creates an aldehyde group at the end of a heparin fragment, which is thought to attach covalently via end-point attachment to the PEI spacer. The attachment may be at least partially ionic, although this surface does not leach heparin in the presence of whole blood.[23] This process creates a surface called the Medtronic/Carmeda Bioactive Surface ([BAS], Medtronic Cardiopulmonary, Anaheim, CA), which is available in a disposable circuit with a Medtronic Maxima membrane oxygenator. This heparin-bonded oxygenator circuit costs $1,200 to $1,500, compared to $600 to $700 for the same circuit without the heparin treatment. This particular process has been called a "universal surface."[33]

The other commercially available product differs by ionically binding conventional unfractionated porcine mucosal heparin to a carrier first, then rinsing the extracorporeal surfaces with this heparin-carrier complex. The precise formula for the carrier remains proprietary, but it has been described as alkylbenzyldimethylammonium chloride containing an 18-carbon length alkyl chain.[45] Binding heparin to the carrier makes the molecular complex sufficiently hydrophilic to bind to a variety of synthetic surfaces. This material is called Duraflo II heparin (Bentley Laboratories, Division of Baxter Healthcare Corp, Irvine, CA), and is available on Bentley Univox membrane oxygenator surfaces. This process has been called a "universal coating."[33] The cost of a Duraflo II–treated membrane oxygenator circuit is approximately $800, compared to approximately $600 for the same circuit without heparin coating. Both animal and human studies detailed below reflect considerable experience with these two heparin-binding processes.

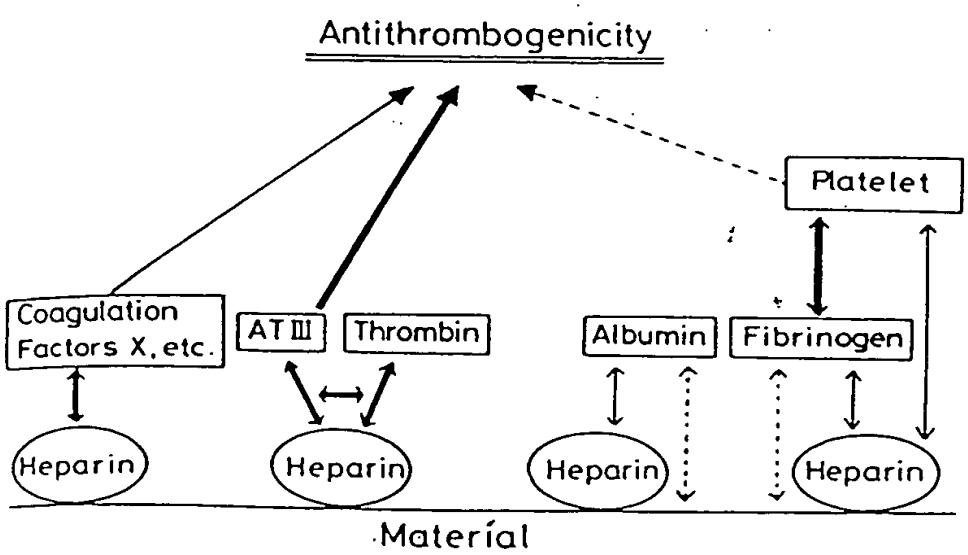

Fig 2. Schematic diagram of interactions at a heparin-coated surface. The relative importance to antithrombogenicity is shown by the type of unidirectional solid arrow: solid wide, strong contribution; solid narrow, medium contribution; dashed, weak contribution. Bidirectional arrows depict interactions, with the strength of the interaction decreasing in the following order: wide solid arrow, narrow solid arrow, dashed arrow. Note that albumin and fibrinogen bind to the surface even in the absence of heparin. (Reprinted with permission.[37])

RECENT ANIMAL STUDIES AND EX VIVO SIMULATIONS

Animal and ex vivo models have extensively compared the hematologic alterations induced by extracorporeal circulation using traditional and heparin-coated circuits. Table 1 summarizes these findings in studies evaluating perfusion times of 6 hours or less. Differences were reported only if they reached statistical significance. Most of these studies investigated small numbers of animals, in many instances fewer than 10. As a result, these studies have low statistical power; therefore, valid and substantial differences might not be unveiled. From these studies, it appears likely that heparin-coated circuits reduce blood trauma, as reflected by the frequent findings of better platelet and leukocyte preservation and less hemolysis (Fig 3). The Carmeda and Duraflo II circuits both performed acceptably. These studies also suggest that reduced levels of systemic anticoagulation are safe with heparin-coated circuits,[46,49,51,53] and possibly that they require no systemic anticoagulation.[38,52,54,55] Three of four studies investigating the latter possibility involved just left heart bypass, which would likely pose a lower risk for thrombogenesis than full CPB. Whittlesey et al[38] found that even uncoated CPB circuits could be safely managed at high flows for a 3-hour period without systemic anticoagulation. However, von Segesser et al[52,57] found that uncoated CPB and left heart bypass circuits developed macroscopic thrombi in the absence of systemic anticoagulation. The same group of investigators commented that less bleeding in the surgical field occurred when heparin-coated circuits were used with reduced or absent systemic anticoagulation, although this observation was not quantified.[52,57]

Many animal studies have also evaluated the hematologic consequences of perfusion with heparin-coated circuits for longer periods ranging from 21 hours to 5 days.[45,58-63] Most of them evaluated heparin-coated circuits used for venovenous bypass without systemic anticoagulation. They found unchanged or moderately abnormal hematologic parameters, including individual coagulation factor levels, antithrombin III levels, platelet counts, leucocyte counts, clotting times, and plasma hemoglobin levels.[45,58-61,63] Two studies found small clots in stagnant zones such as the heat exchanger.[45,62] Only two studies compared heparin-coated circuits to uncoated ones, finding higher plasma hemoglobin levels or lower platelet counts with the uncoated circuits.[45,63] Just as with shorter perfusion periods, satisfactory and practically indistinguishable results were obtained with the Carmeda and Duraflo II surfaces. As yet, no studies have prospectively compared these two circuits in animals or humans.

Three studies have evaluated the inflammatory response to 2-hour perfusion with Carmeda BAS circuits.[64-66] Two of these studies found decreased complement activation with the heparin-coated circuits.[64,65] Videm et al[65] used human blood in an ex vivo simulation to evaluate this response, and used the same model to investigate granulocyte activation with heparin-coated circuits.[66] The latter investigation revealed greater myeloperoxidase release with uncoated circuits, but no difference between the two circuits in lactoferrin release or neutrophil counts.[66]

The renal and blood gas exchange effects of heparin-coated circuits have also been compared to those of uncoated circuits. Weiss et al[56] found higher creatinine clearance and more negative free-water clearance in dogs perfused for 6 hours with Duraflo II heparin-coated circuits, but little or no difference between coated and uncoated surfaces in aldosterone, vasopressin, and natriuretic hormone concentrations. The differences tended to occur toward the end of the perfusion period, and may have resulted from lower mean arterial pressures in the dogs perfused with uncoated circuits. Several studies, most by

Table 1. Hematologic Findings in Animal and In Vitro Studies With CPB for 6 Hours or Less

Author	Surface Type	Model	Bypass Type	Comparison	Principal Findings
Bagge[46]	C	Pig	CPB	SH, CL, CH	Coated circuits had higher platelet count, better platelet adhesion, better leukocyte preservation, and less hemolysis
Palatianos[47]	D	Pig	CPB	SH, CH	No difference in blood loss or platelet count preservation
Palatianos[48]	D	Pig	CPB	CH, CNI	Better platelet count preservation with CNI
Thelin[49]	C	Pig	CPB	SH, CL	Coated circuits showed better platelet and leukocyte preservation and less blood loss, hemolysis, and fibrinolysis
Tong[50]	D	Cow	CPB	SH, CH	Better platelet preservation, less leukocytosis, and less clotting activation with coated circuit
von Segesser[51]	C	Cow	CPB	SH, CL	No difference in hemoglobin, platelet counts, or thrombin time; no macroscopic thrombi in circuits
von Segesser[52]	D	Dog	CPB	SH, CN	Less hemolysis with coated circuit
von Segesser[53]	C	Cow	CPB	SH, CNA	No differences in hemolysis, platelet counts, or thrombin times
von Segesser[54]	C	Cow	LH	SN, CN	No differences in hemoglobin or platelet counts, 1 of 5 SN devices clotted, more emboli and thrombin with SN
von Segesser[55]	C	Cow	LH	SN, CN	No differences in hemolysis, platelet depletion, or macroscopic thrombi
Weiss[56]	D	Dog	LH	SH, CN	Less hemolysis with coated circuit
Whittlesey[38]	O	Rabbit	CPB	SH, SN, CN	No differences in thrombosis, emboli, or platelet counts in 5-hour perfusion

Abbreviations: C, Carmeda Bioactive surface; D, Duraflo II surface; O, other; CPB, full cardiopulmonary bypass; LH, left heart bypass; SH, standard, uncoated circuit with traditional high-dose systemic heparin; SN, standard circuit with no systemic heparin; CH, heparin-coated circuit with traditional high-dose systemic heparin; CL, heparin-coated circuit with low-dose systemic heparin; CN, heparin-coated circuit with no systemic heparin; CNA, heparin-coated circuit with no systemic heparin but with high-dose systemic aprotinin; CNI, heparin-coated circuit with no systemic heparin but with systemic Iloprost for platelet preservation.

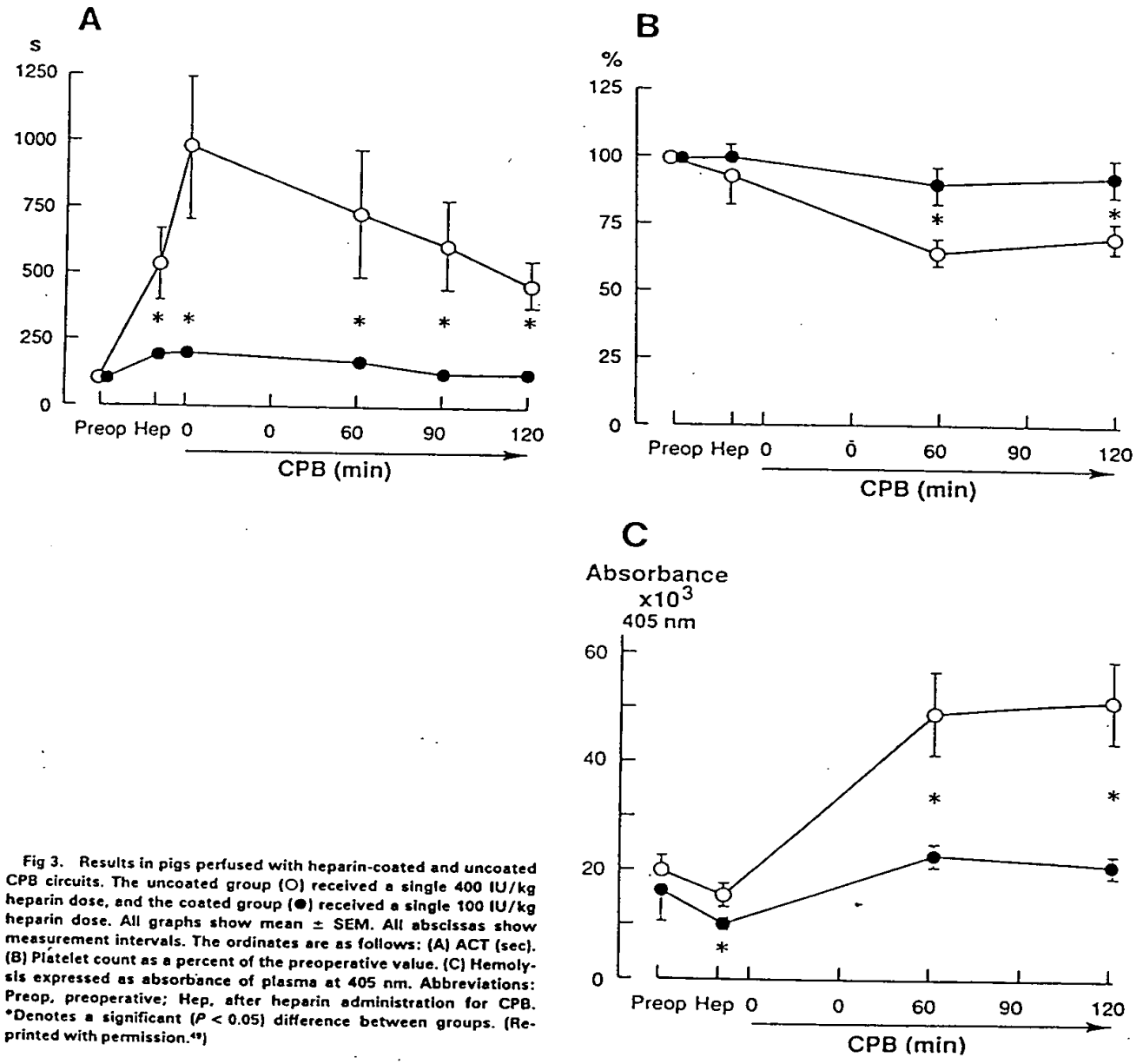

Fig 3. Results in pigs perfused with heparin-coated and uncoated CPB circuits. The uncoated group (○) received a single 400 IU/kg heparin dose, and the coated group (●) received a single 100 IU/kg heparin dose. All graphs show mean ± SEM. All abscissas show measurement intervals. The ordinates are as follows: (A) ACT (sec). (B) Platelet count as a percent of the preoperative value. (C) Hemolysis expressed as absorbance of plasma at 405 nm. Abbreviations: Preop, preoperative; Hep, after heparin administration for CPB. *Denotes a significant ($P < 0.05$) difference between groups. (Reprinted with permission.[49])

the same group of investigators, have found satisfactory and similar arterial blood gases with both heparin-coated and uncoated circuits.[38,45,51-53] von Segesser et al[57,67] found higher mixed venous O_2 saturations when using heparin-coated circuits for left heart bypass, which probably resulted from the higher cardiac output and lower blood loss in the animals perfused with coated circuits.

HUMAN REPORTS AND STUDIES

The Carmeda Bioactive and the Duraflo II heparin-coated surfaces were recently introduced. Each offers a surface coating with greater stability than those used briefly in the 1970s. In 1987 Bindslev[68] used the Carmeda surface for extracorporeal carbon dioxide elimination to treat a patient with acute respiratory failure. All but one of the other clinical reports using these circuits were published in 1990 or later. Table 2 lists the clinical applications that have been published, most of which describe the use of heparin-coated circuits either for cardiac surgery or for pulmonary support.

Table 2. Clinical Applications Reported for Heparin-Coated Extracorporeal Circuits

Cardiac surgery
Pulmonary support
Right or left ventricular mechanical support
Partial bypass for descending thoracic aneurysm repair
Resuscitation of accidental hypothermia
Cerebral aneurysm surgery

CARDIAC AND DESCENDING THORACIC AORTIC SURGERY

Table 3 details most of the reported experience in cardiac surgical patients. Studies have primarily compared hematologic responses to CPB, consisting of bleeding outcomes, or of chemical evidence for activation of the plasma coagulation cascade, the complement system, or leukocytes. No consistent pattern has emerged from these studies, all of which investigated patients undergoing coronary revascularization. The inconsistencies among studies may result from differences in the two coating techniques, differences in systemic anticoagulation, or from group sizes too small to detect statistically significant differences with acceptable statistical power. Only von Segesser et al[76,77] have tested heparin-coated circuits with systemic anticoagulation levels below those conventionally used for CPB in humans, and their results appear promising with respect to reducing post-CPB blood loss (Fig 4). von Segesser et al also used heparin-coated circuits with limited systemic anticoagulation during partial CPB in 12 patients undergoing resection of descending thoracic or thoracoabdominal aortic aneurysms[77] and three Jehovah's Witnesses undergoing myocardial revascularization.[78] Bennett et al[79] used a similar approach in two descending thoracic aortic aneurysm repairs and in one traumatic descending thoracic aortic transection repair, and avoided heparin altogether in one patient undergoing repair of a traumatic descending thoracic aortic transection. In order to safely manage CPB using ACTs as low as 180 seconds, von Segesser et al[76,77] avoided placing a cardiotomy reservoir in the extracorporeal circuit, because heparin coating was not available for that surface. That surface can now be coated with heparin, which will greatly simplify future investigations using reduced levels of systemic anticoagulation (L.C. Hsu, personal communication, September 1993). At this point, it can only be concluded that heparin-coated surfaces are at least as hematologically safe as uncoated ones for CPB in humans, and that some studies show reductions in clotting abnormalities or the inflammatory response.

Two studies have compared neurologic parameters in patients undergoing myocardial revascularization with either heparin-coated or uncoated circuits. Pradhan et al[74] found no difference in retinal microembolization, and Stump et al[80] found no differences in cerebral blood flow during CPB or in neurologic or neuropsychologic outcomes after CPB. Both of these studies used identical and traditional systemic anticoagulation protocols for the coated and uncoated extracorporeal circuits.

Pulmonary Support

The use of extracorporeal ventilation for acute respiratory failure (ARF) proved unsuccessful in the 1970s.[32] Subsequent experience, however, indicated that this failure probably resulted from overly stringent selection criteria.[81] Intervening with extracorporeal life support before pulmonary damage was near-terminal improved survival from ARF, possibly by reducing the exacerbation of pulmonary pathology induced by mechanical ventilation before the disease became irreversible.[82] Because patients with ARF often have complicating conditions such as head injuries, stress ulcers, and disseminated intravascular coagulation, the need to anticoagulate patients undergoing extracorporeal ventilation raises concern about increasing the risk of hemorrhagic complications. For this application of extracorporeal circulation, a nonthrombogenic surface therefore offers a compelling theoretical advantage. The advantage has apparently been so compelling that there have been no prospective human comparisons of heparin-coated and uncoated surfaces in this clinical setting. Rossaint reported 26 patients, 13 of whom satisfied entry criteria for extracorporeal lung assistance (ECLA).[83] Eleven of the 13 conventionally treated patients never satisfied these criteria, and all 11 of those patients survived. The two remaining patients in the conventional treatment group satisfied the entry criteria but refused ECLA, and both of those patients died. The 8 of 13 ECLA patients who survived were sicker than all but the two fatalities in the conventional treatment group. A survival benefit with ECLA using a heparin-

Table 3. Hematologic Comparisons in Prospective Human Studies Comparing Heparin-Coated and Uncoated CPB Circuits for Cardiac Surgery

Study	Surface Type	Number of Patients	ACT Threshold*		Principal Findings
			Coated	Uncoated	
Borowiec[69]	C	14	300	400	No difference in bleeding or transfusions
Borowiec[70]	C	14	300	400	Lesser myeloperoxidase and lactoferrin increases in coated group
Fosse[71]	C	20	300	480	Less increase in complement in coated group
Gravlee[9]	D	22	400	400	No differences in bleeding filter debris, FPA, or BTG
Gu[11]	D	30	400	400	No differences in FPA or AT III levels; early CPB TAT levels less in coated group
Gu[72]	D	30	400	400	Less increase in post-protamine C3a elastase, and TNF in coated group
Mollnes[73]	C	20	300	480	No difference in complement activation
Pradhan[74]	D	14	?†	?†	Lesser TAT increase late in CPB in coated group; no differences in bleeding, complement activation, elastase, or pulmonary neutrophil sequestration
Videm[75]	C	20	300	480	Less complement activation in coated group
von Segesser[76]	D	12	180	480	Less bleeding and transfusion in coated group
von Segesser[77]	D	22	180	480	Less bleeding and transfusion, lower D-dimer levels in coated group

NOTE. All studies include only patients undergoing coronary revascularization.

*ACT level (sec) at which additional heparin was administered. Lower ACT thresholds were also accompanied by smaller initial heparin doses.

†Both groups received "conventional heparin dosing." ACT level not specified.

Abbreviations: ACT, activated clotting time; FPA, fibrinopeptide A; BTG, betathromboglobulin; AT III, antithrombin III; TAT, thrombin-antithrombin III complex; TNF, tumor necrosis factor; C, Carmeda BioActive Surface; D, Duraflo II heparin-coated surface.

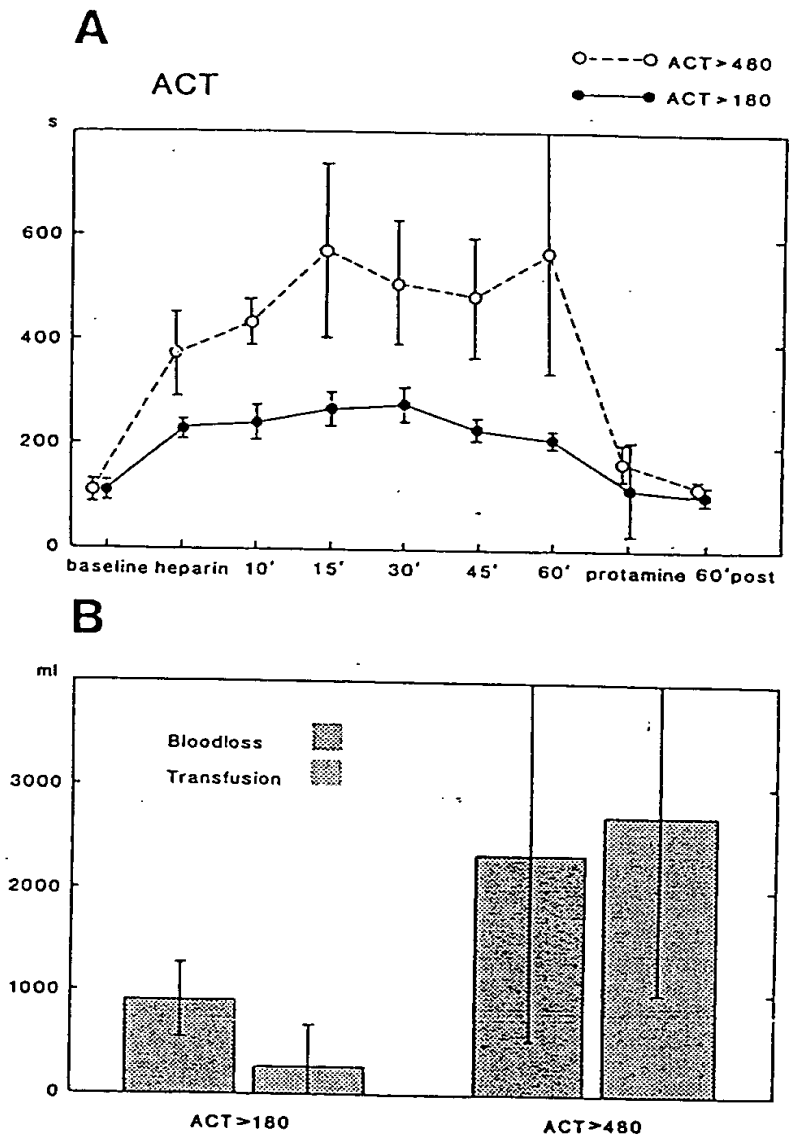

Fig 4. A comparison between low-level and high-level systemic anticoagulation in patients undergoing coronary revascularization with heparin-coated circuits. (A) ACT values (mean ± standard deviation) before, during, and after bypass. (B) Total blood loss and transfusion (mean ± standard deviation) in the low-heparin (ACT > 180) and high-heparin (ACT > 480) groups. The differences between groups were significant for both blood loss ($P < 0.01$) and transfusion ($P < 0.001$). (Reprinted with permission.[77])

coated surface compared with conventional mechanical ventilation may thus be inferrred.

Several case reports and two series using heparin-coated surfaces for ECLA have been reported,[68,79,83-88] all of which have used the Carmeda BioActive Surface. The majority of these reports have used venovenous bypass, with a duration of pulmonary support ranging from 2 hours to 40 days. One of the two series is described above,[83] and the other reported survival in four of seven patients treated for pulmonary insufficiency.[79] Rossaint et al[83] did not provide information about the duration or quality of survival, whereas Bennett et al[79] defined survivors as patients living 60 or more days after bypass termination; most of whom had returned to their "pre-bypass activities" (Did these "activities" include endotracheal intubation and mechanical ventilation?). Among the several case reports, survival after ECLA was reported in five of six patients,[68,84-88] the longest follow-up being 4 months. The patient reported by Peters et al[85] did not survive weaning from ECLA.

In most of these reports, the extracorporeal circuit primarily augmented carbon dioxide elimination. Continued mechanical ventilation at lesser frequencies and mean airway pressures provided oxygenation via the diseased lungs largely by apneic oxygen diffusion.[68] Because patients required little extracorporeal oxygenation and no circulatory support, the flows through the extracorporeal circuit usually remained less than 2 L/min. This would likely vary with the severity of the pulmonary disease. Either roller pumps or impeller-type pumps can be used.

Whereas pulmonary support with uncoated circuits traditionally uses a true membrane oxygenator made of silicone, the heparin-coated circuits have only been available with hollow-fiber semipermeable membrane oxygenators. Semipermeable membranes tend to leak plasma with prolonged

use, which progressively induces an irreversible state of "oxygenator pulmonary edema" that requires changing to a new oxygenator. Rossaint et al[83] used 113 oxygenators in 227 days of clinical use, and Wetterberg and Steen[87] used 15 oxygenators over 35 days.

When ECLA uses a traditional uncoated circuit, most centers have infused heparin to maintain the ACT between 170 and 240 seconds. Should bleeding ensue or surgery be required, ACTs range from 150 to 180 seconds, although some have reported oxygenators clotting in this range.[81] Most investigators using heparin-coated circuits for ECLA use low levels of systemic anticoagulation induced by a continuous heparin infusion, usually to an ACT below 200 seconds or an activated partial thromboplastin time below 50 seconds.[68,85,87,88] Rossaint et al[83,86] maintained the ACT below 150 seconds. In some reports, anticoagulation was either never used or was discontinued for reasons such as multiple trauma,[84] the need for surgery,[83,86] or the presence of bleeding.[87] In the case reported by Wetterberg, no systemic anticoagulation was used for 23 of the 35 days of ECLA.[87] Rossaint et al[83,86] supplemented anticoagulation with prostacyclin for platelet inhibition and with antithrombin III concentrate to maintain plasma antithrombin III concentrations at least 80% of normal. Wetterberg and Steen[87] used antithrombin III concentrates and plasma to maintain plasma antithrombin III levels at least 100% of normal. Macroscopic clots in the extracorporeal circuit did not occur in any of the patients receiving ECLA with heparin-coated circuits. Two circuits were washed with saline after ECLA termination and found to have minimal fibrin formation either macroscopically[84] or by scanning electron microscopy.[85]

Other Clinical Uses

Limited experience has been reported with heparin-coated surfaces for resuscitation of accidental hypothermia[77,79,89] and for support of a failing right or left ventricle after CPB.[79,90] One of three hypothermic patients survived.[79,89] Two of these three resuscitations with heparin-coated circuits did not utilize systemic anticoagulation. Bennett et al[79] used the Carmeda surface, and von Segesser et al[89] used the Duraflo II surface.

Bennett and colleagues[79] used closed-chest cardiopulmonary support in two patients who underwent cardiac surgical procedures, both of whom received limited systemic anticoagulation with the Carmeda surface and survived. Saito et al[90] treated 13 patients experiencing profound post-CPB cardiac failure with ventricular assistance (right-, left-, or bi-ventricular) using a heparin-coated surface (Anthron) and a roller pump-driven system. Systemic anticoagulation, if used, was not reported. Eight patients were successfully weaned from this support, and six survived to be discharged from the hospital.

Bennett et al[79] used closed-chest venoarterial bypass to accomplish deep hypothermic circulatory arrest for a patient undergoing clipping of a cerebral aneurysm. Limited heparin doses were administered to maintain ACTs between 121 seconds and 262 seconds over a bypass period of 3 hours and 17 minutes. The patient survived.

SUMMARY

The indications for heparin-coated extracorporeal circuits cannot be defined or limited at present. Clinical investigation remains at an early stage of development. In situations where the risk of systemic anticoagulation is high, this technology would seem to hold great promise. Examples include extracorporeal lung assist and resuscitation from accidental hypothermia. Some have also suggested the use of heparin-coated circuits for percutaneous bypass in cardiopulmonary resuscitation.[91] A significant advantage might also accrue in noncardiac surgical procedures requiring cardiopulmonary bypass, such as complex cerebral aneurysm or arteriovenous malformation resections, resections of the tracheal carina, or bilateral lung transplantations. Its role in routine cardiac surgical procedures remains uncertain, but the work of von Segesser et al[76,77] suggests a need for continued investigation in that setting using reduced levels of systemic anticoagulation. That endeavor will be greatly assisted by the recent development of heparin-coated cardiotomy reservoirs. Although heparin-coated circuits have been safely used for extracorporeal lung assist with little or no systemic anticoagulation, prospective studies are clearly needed to determine if this approach is advantageous, and it would seem appropriate to develop heparin coating for silicone-based membrane oxygenators.

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Synthesis of phospholipid polymers having a urethane bond in the side chain as coating material on segmented polyurethane and their platelet adhesion-resistant properties

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Surface modification of segmented polyurethanes (SPUs) was carried out using new blood compatible polymers having both phospholipid polar groups and urethane bonds in the side chains. The polymers were composed of 2-methacryloyloxyethyl phosphorylcholine (MPC), *n*-butyl methacrylate (BMA) and methacrylate with a urethane bond (MU). The MPC copolymers were soluble in ethanol. The SPU membranes were immersed in an ethanol solution of MPC copolymers and dried *in vacuo* for coating. The surface formed was completely covered with the MPC copolymer which was confirmed by X-ray photoelectron spectroscopic analysis. The polymer coatings were hardly detached in water, ethanol and 40% aqueous solution of ethanol compared with poly(MPC-*co*-BMA) which did not have the MU moieties. Therefore, the MU moieties had affinity for the SPU. The surface modification of the SPUs suppressed platelet adhesion effectively after contact with platelet-rich plasma for 180 min.

Keywords: Polyurethanes, haemocompatibility, phospholipid polymer, platelet adhesion, surface modification

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Segmented polyurethanes (SPUs) are widely used as biomedical materials because of their excellent mechanical properties and blood compatibility[1]. The hard segments consist of urethane or urea linkage forming domains which disperse in the matrix of the soft segments. The primary driving force for the domain formation is the strong intermolecular interaction between the urethane or urea units, which are capable of forming hydrogen bonds.

Lyman *et al.*[2,3] investigated the blood compatibility of these SPUs to clarify their structural effects on protein adsorption and platelet adhesion. Because these SPUs form a microphase-separated structure composed of two incompatible segments, they assumed that these segmented copolymers have two-phase morphologies with a domain of size 3–10 nm, which approximates to the size of globular proteins. The surface composition of SPU shows very important and interesting effects on platelet adhesion. Based on X-ray photoelectron spectroscopic observation of SPU, Sung and Hu[4] estimated the concentration of soft segments at the surface, and found a linear correlation between the surface concentration of soft segments and platelet adhesion on the surface. However, the blood compatibility of the SPUs was not satisfactory for long-term implantation as a vascular graft of small diameter. Moreover, Zhao *et al.*[5] reported the degradation of the SPU by adsorption of proteins, adhesion of macrophages and peroxide formation. The soft segment, which was mainly a polyether chain, was degraded by the oxygen radicals produced by the macrophages[5]. Therefore, it is necessary to reduce cell adhesion to prevent degradation of the SPU when it is used *in vivo* for long periods.

Recently, Nojiri *et al.*[6] reported that the surface of vascular grafts made with the SPU Biomer™ could be modified by coating with poly[2-hydroxyethyl methacrylate (HEMA)-*block*-styrene(St)] from solution. The total occlusion time was significantly elongated, and the thickness of the adsorbed protein layer at the interface was thinner than that on Biomer or on that grafted with poly(ethylene oxide).

In a previous paper[7], we showed that copolymers of 2-methacryloyloxyethyl phosphorylcholine (MPC) with hydrophobic alkyl methacrylates, particularly *n*-butyl methacrylate (BMA), showed excellent non-thrombogenicity even when the poly(MPC-*co*-BMA)s

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were in contact with human whole blood in the absence of an anticoagulant. The poly(MBC-*co*-BMA)s could reduce protein adsorption from plasma[8,9]. Because the MPC copolymers have a phosphorylcholine group which is a typical phospholipid polar group, the copolymers have a strong affinity for phospholipids[10,11]. The phospholipid molecules were adsorbed on the surface of the MPC copolymer from the plasma, resulting in the formation of a 'self-assembled biomimetic membrane' constructed from adsorbed phospholipid molecules and MPC moieties on the surface[7]. The self-assembled biomimetic membrane surface can interact weakly with blood cells and proteins, and does not induce activation of these components. This was confirmed when poly(MPC-*co*-BMA) was treated with dipalmitoylphosphatidylcholine liposomal solution; both protein adsorption and platelet adhesion were suppressed more effectively compared with that observed on the original MPC polymer, even when the mole fraction of MPC in the polymer was low[9,10].

In this study, based on the fundamental properties of poly(MPC-*co*-BMA), the chemical structure of poly(MPC-*co*-BMA) was modified as a coating material suitable for SPUs by the introduction of a urethane bond in the side chain. The effects of the urethane bond introduced into the modified poly(MPC-*co*-BMA) on the affinity for typical SPUs for medical grade use and on platelet adhesion-resistant properties are discussed.

EXPERIMENTAL DETAILS

Materials

2-Methacryloyloxyethyl phosphorylcholine (MPC) was synthesized by a method reported previously[12]. *n*-Butyl methacrylate (BMA, Nakarai Tesque Co., Tokyo, Japan) and 2-isocyanate ethyl methacrylate (IEMA, Kokusan Kagaku Co., Tokyo, Japan) were reagent grade and were purified by vacuum distillation; fractions of b.p. 68.5°C/30 mm Hg and b.p. 60.0°C/2.5 mm Hg were used, respectively. Toluene was distilled over calcium hydrate, and the fraction of b.p. 110.6°C was used. *n*-Butanol and benzyl alcohol were used after drying with molecular sieves 4A. Two kinds of SPUs were used in this study. Poly(ether urethane), Pellethane" 2363-90 (PT-90, Upjohn Co.), was kindly supplied by Terumo Co. Ltd. Poly(ether urethaneurea), TM-3™, was kindly given by Toyobo Co. as a 10 wt% solution in *n,N*-dimethylacetamide (DMAC). The structures of these SPUs are shown in *Figure 1*. Other reagents were commercially available in extra-pure grade and were used without further purification.

Monomer synthesis

Methacrylates with a urethane bond in the side chain (MUs) were synthesized by the reaction between IEMA and the corresponding alcohol or phenol[13,14]. The chemical formulae of these monomers are shown in *Figure 2*. The general synthetic procedure for MU was as follows. A 300 ml three-necked flask equipped with a condenser, a thermometer and a magnetic stirrer was loaded with 0.1 mol of IEMA, 0.105 mol of the alcohol or phenol, and a small amount of dibutyltin dilaurate (DBTL) as catalyst. Into this mixture, 150 ml of toluene were added as a solvent. The solution was stirred at 60°C for a given time. After the reaction, the toluene was removed using an evaporator in the case of 2-methacryloyloxyethyl butylurethane (MEBU) synthesis, and the mixture was distilled under reduced pressure. In the cases of 2-methacryloyloxyethyl benzylurethane (MEBZU) and 2-methacryloyloxyethyl phenylurethane (MEPU), the reaction mixture was cooled in a refrigerator to crystallize the products. The structure of the MUs obtained was confirmed by ^{1}H NMR spectroscopy, IR spectroscopy and elemental analysis. Spectral and thermal data for each methacrylate are shown as follows. MEBU: ^{1}H NMR:($CDCl_3$, σ in ppm) 0.83–0.88 (α-C$\underline{H}_3$, 3H), 1.26–1.55 (C$\underline{H}_2$ and C$\underline{H}_3$ in side chain, 7H), 3.40–3.43 (C$\underline{H}_2$–N, 2H), 3.95–4.05 (NCOO–C$\underline{H}_2$, 2H), 4.10–4.17 (CCOO–C$\underline{H}_2$, 2H), 4.80–4.95 (–NHCOO–, 1H), 5.52–5.53 (C=C$\underline{H}$, 1H), 6.05–6.06 (C=C$\underline{H}$, 1H); IR: (cm^{-1}) 3200–3400 (–NCOO–), 2800–3000 (C$\underline{H}_2$ and C$\underline{H}_3$), 1720 (C=O), 1640 (C=CH_2), 1580 (–NHCOO–), 1100–1260 (–COO–); b.p. = 132–135°C/2 mm Hg. MEBZU: ^{1}H NMR: ($CDCl_3$, σ in ppm)

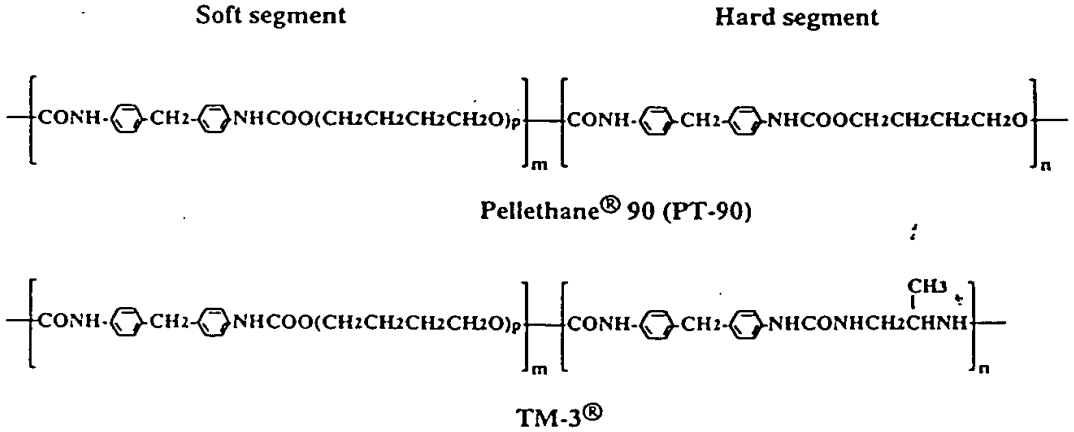
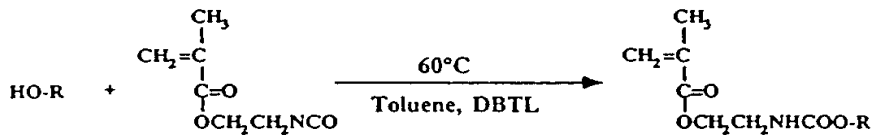
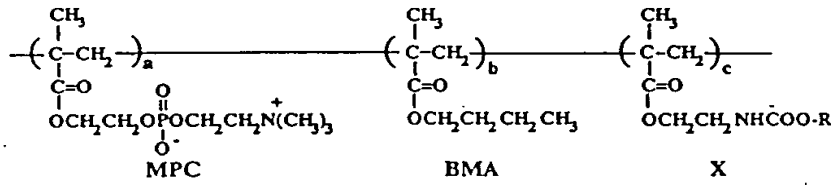


Figure 1 Chemical structure of the segmented polyurethane (SPU) used in this study.



R	Abbreviation
$-(CH_2)_3CH_3$	MEBU
$-CH_2-C_6H_5$	MEBZU
$-C_6H_5$	MEPU

Figure 2 Synthetic route of methacrylate having a urethane bond in the side chain (MU).



R	Abbreviation
$-(CH_2)_3CH_3$	PMBBU
$-CH_2-C_6H_5$	PMBBZU
$-C_6H_5$	PMBPU

Figure 3 Chemical structure of MPC copolymers.

1.85–1.90 (α-CH_3, 3H), 3.44–3.45 (CH_2-N, 2H), 3.95–4.05 (NCOO–CH_2, 2H), 4.15-4.17 (CCOO–CH_2, 2H) 4.95–4.98 (–NHCOO–, 1H), 5.00–5.04 (–NCOCH_2–, 2H), 5.50–5.51 (C=CH, 1H), 6.00–6.03 (C=CH, 1H), 7.17–7.29 (C_6H_5–, 5H); IR: (cm^{-1}) 3250–3350 (–NHCOO–), 2800–3000 (CH_2 and CH_3), 1720 (C=O), 1640 (C=CH_2), 1600 (aromatic ring), 1580 (–NHCOO–), 1100–1260 (–COO–); m.p. = 35°C (waxy at room temperature[13]). MEPU: ^{1}H NMR: ($CDCl_3$, σ in ppm) 1.97 (α-CH_3, 3H), 3.57–3.60 (CH_2-N, 2H), 4.29–4.31 (CCOO–CH_2, 2H), 5.35 (–NHCOO–, 1H), 5.61–5.62 (C=CH, 1H), 6.16 (C=CH, 1H), 7.11–7.37 (C_6H_5–, 5H); IR: (cm^{-1}) 3280–3380 (–NHCOO–), 2800–3000 (–CH_2– and –CH_3), 1720 (C=O), 1640 (C=CH_2), 1580 (–NHCOO–), 1500 (aromatic ring), 1100–1260 (–COO–); m.p. = 110°C (108°C[13], 106°C[14]).

Copolymerization

The desired amounts of MPC, BMA and one of the MUs were placed in a glass ampoule, and the mixture was diluted with ethanol to 1 mol l^{-1} of monomer concentration. Into this solution, 2,2′-azobisisobutyronitrile (AIBN) was dissolved (1 mmol l^{-1}). Argon was bubbled into the solution to displace the oxygen, and then the ampoule was sealed. The polymerization was carried out at 60 C for a given period. After cooling, the contents were poured into a large amount of a mixture of diethyl ether and *N,N*-dimethylformamide (DMF) (9/1 by volume) to remove unreacted monomer and precipitate the polymer formed. The precipitate was filtered off and dried *in vacuo*. The structure of the copolymers obtained was confirmed by ^{1}H NMR, IR and elemental analysis. The mole fraction of each component in the copolymer was determined from the results of phosphorus analysis for the MPC units and nitrogen analysis for both the MPC and MU units. The chemical structure of these copolymers and results of the copolymerization are shown in *Figure 3* and *Table 1*, respectively.

Table 1 Synthesis of the MPC copolymer having a urethane bond in the side chain

Abbrev.	Mole fraction (MPC/BMA/X)		Time[a] (h)	Yield (%)	Mw[b] ($\times 10^4$)
	in feed	in copolymer			
PMB	0.30/0.70/0.00	0.31/0.69/0.00	15	53.8	4.7
PMBBU	0.30/0.60/0.10	0.36/0.53/0.11	4	40.8	3.8
PMBBZU	0.30/0.60/0.10	0.26/0.63/0.09	4	55.6	5.9
PMBPU	0.30/0.60/0.10	0.30/0.58/0.12	2.5	43.9	4.1

[a][Monomer] = 1.0 mol l^{-1} and [AIBN] = 1.0 mmol l^{-1} in ethanol. Polymerization temperature = 60 C.
[b]Weight-averaged molecular weight (Mw) was determined by GPC with poly(St) standard (eluent = DMF/tetehanol 9(1)).

Preparation of polymer membrane and coating of the MPC copolymer

The SPU membranes were prepared by a solvent evaporation technique. The PT-90 was dissolved in tetrahydrofuran (THF) and a 10 wt% solution was prepared. The THF solution was spread on a Teflon® plate, and the THF was evaporated at room temperature. The membrane formed was dried under reduced pressure at 40°C to remove the residual solvent. The same procedure was applied to membrane processing of TM-3 using a 10 wt% solution in DMAc. The thickness of the membrane obtained was 300 μm. The MPC copolymer was dissolved in ethanol to make a 0.5 wt% solution. The SPU membranes were immersed in the solution containing MPC copolymer for 1 min and dried overnight at room temperature to evaporate the solvent. After this procedure was repeated twice, the membrane was dried *in vacuo* at room temperature. The surface of the membrane coated with the MPC copolymers was analysed during X-ray photoelectron spectroscopy (XPS, Shimadzu ESCA-750, Kyoto, Japan). The take-off angle of the photoelectron was 60° and the elements detected were carbon (C_{1s}), nitrogen (N_{1s}), oxygen (O_{1s}) and phosphorus (P_{2p}).

Elution test of the MPC copolymer from the membrane[15]

The SPU membranes coated with the MPC copolymer were punched out as discs (15 mm in diameter) and immersed in various solvents for 180 min to elute the MPC copolymer. The solvents for the elution test were distilled water, ethanol and 40 vol% aqueous ethanol solution. A specific amount of these solvents was taken out, and the amount of the MPC copolymer in the solvent was determined by phosphorus analysis. After the membrane was immersed in the solvent, the membrane was dried under reduced pressure for 1 day, and the surface was analysed by XPS to identify the MPC copolymers remaining on the surface. The bar graphs in *Figure 4* represent the mean values of triplicate samples (± standard deviation) of the conditions. A comparative analysis was determined using the analysis of variance and Student's *t*-test.

Blood contacting test

The disc-shaped SPU membranes (15 mm in diameter) were placed into a 24-well culture plate and fixed with a silicone ring. To equilibrate the membrane surface, 1 ml of phosphate-buffered solution (PBS, pH 7.4; ionic strength, 0.15 M) was added into each well and allowed to remain for 15 h. After removing the PBS, 1 ml of platelet-rich plasma (PRP) was poured onto the membrane and allowed to remain at 37°C for a given time. The PRP was removed with an aspirator, and the membrane was rinsed three times with 1 ml of PBS. Then 1 ml of 2.5 vol% glutaraldehyde in PBS was poured into each well, and the materials were maintained at room temperature for 2 h to fix the blood components on the membrane. After rinsing sufficiently with distilled water, the membrane was freeze-dried. The surface of the membrane was observed by scanning electron microscopy (SEM, JEOL JSM-5400, Tokyo, Japan) following gold-sputtering on the membrane surface.

RESULTS

Preparation of MPC copolymers with a urethane bond in the side chain

Three kinds of MUs were synthesized by the reaction between IEMA and the corresponding alcohols or phenol. The ^{1}H NMR and IR spectral data clearly show the chemical structure of these monomers (see Experimental Details section), and elemental analysis of the MUs corresponded to the calculated values. All the MUs synthesized could be dissolved in ethanol, DMF, chloroform and DMAc. Therefore, the MU was copolymerized with MPC and BMA in ethanol as a solvent. The copolymerization proceeded homogeneously, and ternary copolymers were obtained. The composition of each monomer unit in the copolymer was almost the same as that in the feed. The copolymers obtained could be dissolved in ethanol and swollen in water but were insoluble in THF, DMF and DMAc.

Surface characteristics of SPU membrane coated with MPC copolymer

The coating of the copolymer on the SPU membrane was confirmed by XPS. The representative XPS charts of PT-90 alone and PT-90 coated with PMBBU are shown in *Figure 5*. The carbon peaks attributed to the C–O bond at 287 eV and the C=O group at 288 eV showed clearly, and new peaks of nitrogen at 403 eV and phosphorus at 134 eV were observed after the PMBBU coating. After coating the surface of PT-90 with PMBBZU or PMBPU, the new peaks attributed to the nitrogen and phosphorus were also observed.

The ratio of the peak area of phosphorus to that of carbon (P/C) was calculated and is represented in *Figure 4*. The P/C value after immersion in water did not change significantly ($P > 0.01$) compared to the original value for every PT-90 coated with MPC copolymers. However, a significant decrease ($P < 0.01$) in the P/C values was found when the PT-90 coated with PMB which did not contain the MU unit was immersed in ethanol and aqueous ethanol solutions. The decrease in the P/C value was found in the case of the PMBPU coating; however, the decrease in the P/C value was relatively small compared with the PMB coating case. When the surface of TM-3 coated with these MPC copolymers was analysed by XPS, there was no significant change in the P/C value after immersion in every solvent except for the PMB coating. The amounts of the MPC copolymers eluted from the surface in every elution test were too small to be detected quantitatively.

Platelet adhesion on SPU coated with MPC copolymers

Figures 6 and *7* show the SEM pictures of the surface of the original SPU membranes and those coated with the MPC copolymers after contact with PRP for 60 and

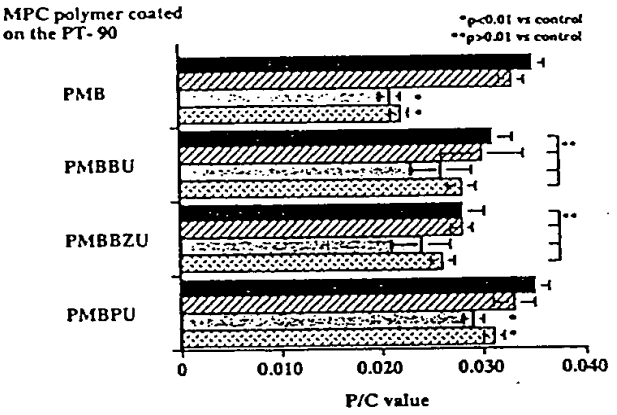


Figure 4 The P/C values determined from the X-ray photoelectron spectra of PT-90 coated with MPC copolymers before and after immersion in various solvents. (▩) Before immersion. After immersion in (▨) water, (▩) ethanol, (▨) 40% aqueous ethanol solution.

180 min. On both original SPU membranes, a lot of platelets adhered. The number of platelets adhered on these SPUs increased with an increase in the contact periods. However, coating the MPC copolymers effectively reduced the number of adherent platelets even when the contact time was increased to 180 min. Moreover, the shape change in the adherent platelets was also suppressed by the MPC copolymer coating even after 180 min contact. On the surface of PT-90 coated with PMBPU, the platelets were observed to adhere in patches without aggregation after 180 min contact with PRP. In the case of TM-3, a difference in the platelet adhesion-resistant properties between the coated MPC copolymers could not be observed.

DISCUSSION

In the biomedical field, SPUs have been used widely because of their excellent mechanical properties[1]. The SPUs assume a so-called 'microdomain structure'; that is, hard segments interact with each other through hydrogen bonds and aggregate in the domains of the soft segments. Some investigators have reported that the SPUs have good blood compatibility such as the reduction of blood cell adhesion due to their microdomain structure[1-3]. However, the amount of proteins adsorbed on the SPU surface was extremely large and cells adhered on the surface.

Our previous articles reported the syntheses of biomedical polymers having phospholipid polar groups, MPC copolymers, and evaluation of the blood compatibility with attention to cell adhesion and protein adsorption *in vitro*[8-11]. We found that poly(MPC-*co*-BMA) could suppress platelet adhesion and activation even in contact with human whole blood in the absence of an anticoagulant[7]. The results could be explained by reduction of the amount of protein adsorbed by the poly(MPC-*co*-BMA). We have suggested that poly(MPC-*co*-BMA) is used as a coating material. The SPUs are generally used under loading conditions of continuous stress and deformation. Therefore, it is necessary to immobilize the coating polymer on the surface to prevent detachment. Although many attempts were made to modify the SPU surface with chemical substances which included polymeric materials, most of these substances were bonded covalently[15-16]. However, chemical modification of the SPU surface is not possible after fabrication of medical devices. We considered that if a urethane bond were introduced into the polymer, especially in the side chain, the polymer would have affinity for the SPU through hydrogen bonding. Therefore, the MUs were synthesized and copolymerized with MPC and BMA. Although some MUs have been reported in the literature and used as photo- or electron-resist materials, they have not been used as biomaterials[13-14]. The copolymerization of these monomers in ethanol proceeded well, and the mole fraction of each monomer unit in the copolymer obtained corresponded closely to that in the feed. The reactivity of these monomers was considered to be almost the same because the monomers were basically derivatives of ethyl methacrylate. Thus, it is very easy to obtain a

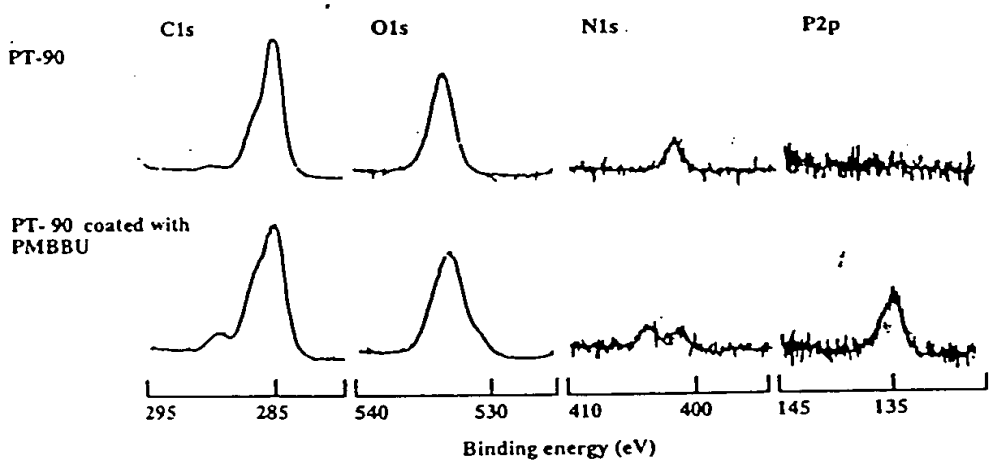


Figure 5 X-ray photoelectron spectra of PT-90, and PT-90 coated with PMBBU.

suitable MPC copolymer having a urethane bond in the side chain.

Previous reports on the blood compatibility of the MPC copolymers indicated that more than 0.25 MPC mole fraction in the copolymer was necessary to obtain satisfactory platelet adhesion- and protein adsorption-resistant properties[7,8,19–22]. The MPC copolymers became hydrated when they were immersed in water but did not dissolve in water[12]. The MU units were expected to act as an anchor to the hard segment located at the surface of the SPU membrane. It is considered that the immobilization of hydrated MPC copolymer can be achieved with a simple coating. In fact, an elution test revealed the effectiveness of the urethane bond in the side chain. The elution of the PMB occurred easily when the SPU membrane coated with PMB was immersed in a solvent containing ethanol. On the contrary, PMBBU and PMBBZU hardly detach even when the SPU membrane coated with these MPC copolymers is

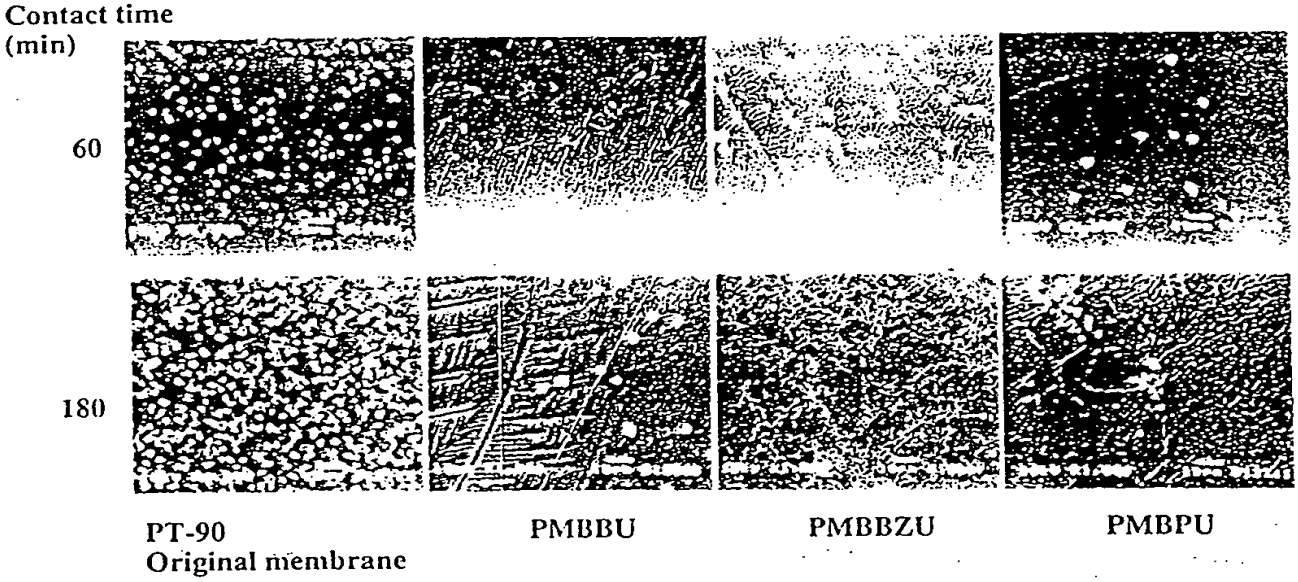


Figure 6 Micrographs of original PT-90 and that coated with MPC copolymers after contact with platelet-rich plasma for 60 and 180 min.

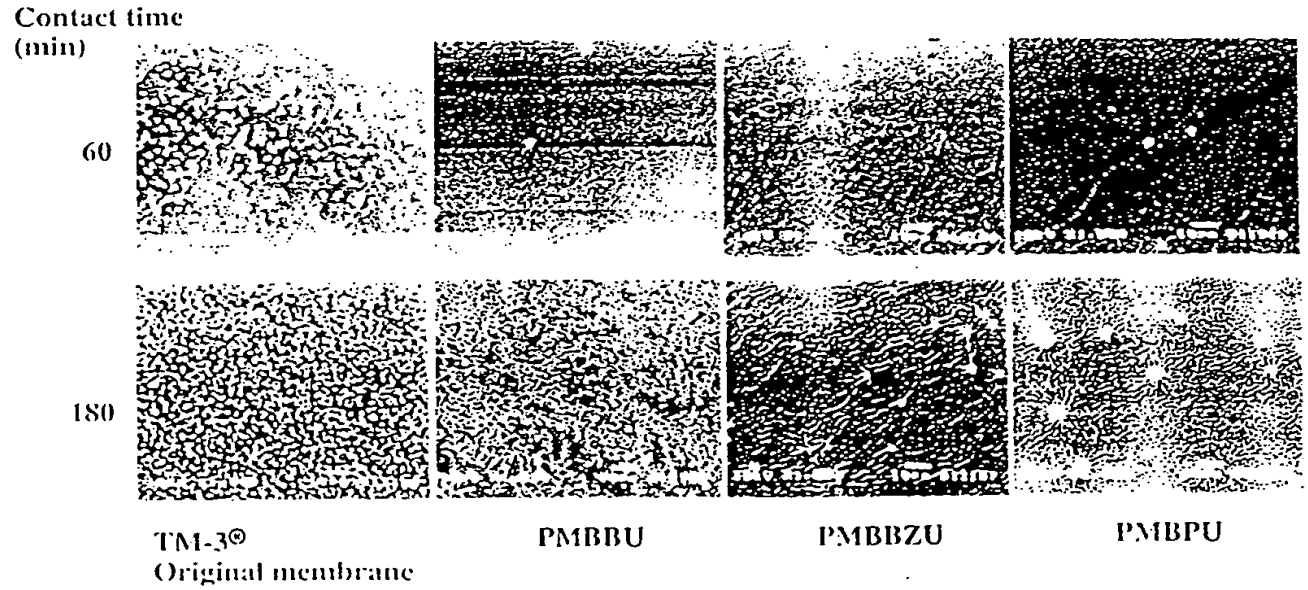


Figure 7 Micrographs of original TM-3© and that coated with MPC copolymers after contact with platelet-rich plasma for 60 and 180 min.

immersed in ethanol, which is a good solvent for the MPC copolymers.

The platelet adhesion of the SPUs was effectively suppressed after coating with the MPC copolymers and did not depend on the contact period up to 180 min. This finding indicated that the MU residues did not show any adverse effects on the platelet adhesion-resistant property of the MPC copolymer. In the case of the SPUs coated with PMBPU, the P/C value slightly decreased on immersion in a solvent containing ethanol, indicating elution of the PMBPU from the membrane. A slight difference in the platelet adhesion-resistant property could be observed; that is, the platelets adhered in patches compared with other MPC copolymers. Therefore, the platelet adhesion was considered to correspond to partial detachment of the PMBPU from the PT-90 surface.

Based these observations, it is concluded that the MPC copolymers having MU units are useful materials for improving the surface blood compatibility of the SPU by a simple coating method using ethanol solutions. The blood compatibility of the SPU membranes coated with the MPC copolymers under continuous stress are now being tested, and the results will be reported in the near future.

ACKNOWLEDGEMENTS

The authors express their appreciation to Dr Atsushi Takahara, Kyushu University, and Dr Tomoko Ueda-Yukoshi, Tokyo Medical and Dental University, for their helpful suggestions, and Dr Shinobu Tanaka, Tokyo Medical and Dental University, for his assistance in the animal experiments. A part of this study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Japan (04205030, 06558122) and Terumo Scientific Foundation; one of the authors (K.I.) would like to express his appreciation for this support.

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Control of contact activation on end-point immobilized heparin: The role of antithrombin and the specific antithrombin-binding sequence

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The uptake and activation of FXII from blood plasma was studied in small-diameter polyethylene tubing, surface-modified by end-point immobilization of heparin. Two preparations of heparin were used to modify the contact-activating properties of the plastic tubing: unfractionated, functionally active heparin and low-affinity heparin, lacking the specific antithrombin-binding sequence and virtually devoid of anticoagulant activity. The uptakes of FXII on the two heparin surfaces were similar. No activated FXII could be demonstrated on the unfractionated heparin surface, whereas on the low-affinity heparin surface nearly all FXII underwent spontaneous activation. The suppression of FXII activation on the unfractionated heparin surface was investigated by using plasma depleted of antithrombin, complement C1 esterase inhibitor, or both. The removal of antithrombin resulted in extensive activation of FXII, whereas the depletion of C1 esterase inhibitor had only a minor effect. Experiments with recalcified plasma showed rapid clot formation during exposure to the low-affinity heparin surface. After depletion of antithrombin, but not complement C1 esterase inhibitor, the recalcified plasma clotted in contact with the unfractionated heparin surface as well. We conclude that antithrombin and the antithrombin-binding sequence in the surface-immobilized heparin are essential for the prevention of surface activation of FXII and triggering of the intrinsic coagulation system.

INTRODUCTION

Exposure of blood to foreign materials or tissues other than the natural endothelial lining of the vascular wall activates the plasma contact system, which includes the proenzymes coagulation factor XII (FXII), prokallikrein, coagulation factor XI (FXI), and the high-molecular-weight kininogen cofactor.[1] The contact system may represent a key function in blood as it appears to be involved in the triggering mechanisms of several defense systems, including the coagulation, kallikrein, fibrinolytic, and complement systems.[2]

Artificial materials and, in particular, negatively charged surfaces cause activation of the contact system.[1] The development of foreign surfaces that do not provoke activation of the contact system therefore represents a major challenge in the field of biomaterials. Surfaces modified with end-point immobilized heparin, contrary to negatively charged materials in general, have shown a remarkable blood compatibility, despite the negative-charge density of the heparin molecules.[3–5] These surfaces effectively inhibit coagulation factor Xa (FXa) and thrombin by an antithrombin-dependent mechanism similar to that established for heparin in the fluid phase.[6,7] In a more recent study, the activation of FXII on a plasma-exposed heparin surface was also shown to be strongly suppressed and the reduced contact activation was demonstrated to be dependent on the high-affinity antithrombin-binding sequence in the immobilized heparin.[8]

A central role in the natural regulation of activated FXII and kallikrein activity in plasma has been attributed to the serine proteinase inhibitor, complement C1 esterase inhibitor, although α_2-macroglobulin and α_1-antiplasmin as well as antithrombin have also been shown to contribute to a limited extent.[9] The aim of the present investigation was to clarify the functional roles of antithrombin and complement C1 esterase inhibitor for the suppression of contact activation, and hence for the thromboresistant properties of the heparin surface.

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The study was performed using normal plasma and plasma preparations artificially depleted of antithrombin or complement C1 esterase inhibitor, or both, and heparin surfaces with or without the specific antithrombin-binding sequence.

MATERIALS

Antithrombin, of human origin for clinical use, with a specific activity of 6.1 IU/mg protein, was obtained from Pharmacia (Stockholm, Sweden). The lyophilized powder was dissolved in water to a stock concentration of 50 IU/ml and the solution was stored at −70°C in 0.3-ml aliquots.

Factor Xa (FXa), of bovine origin (Chromogenix, Mölndal, Sweden), was dissolved in water to an activity of 0.67 nkat/ml (pH 7.4).

Synthetic peptide chromogenic substrates for FXa (S-2765), kallikrein (S-2302) from Chromogenix, were dissolved in water to a final concentration of 0.9 mmol/L and 2.5 mmol/L, respectively, and stored in the dark at 4°C.

Thrombin–antithrombin complex assay kit, Enzygnost-TAT, a sandwich enzyme immune assay, was purchased from Behringwerke (Marburg, Germany).

Complement C1 esterase inhibitor assay kit, immunochrom complement C1 esterase inhibitor reagent kit, a functional complement C1 esterase inhibition assay, was purchased from Immuno AG (Vienna, Austria).

Cephotest, an aqueous suspension of phospholipid (cephalin) and ellagic acid, was purchased from NycoMed A/S (Oslo, Norway).

r-Hirudin, supplied by Pentapharm (Basel, Switzerland) had a specific activity >10,000 antithrombin units/mg protein. The lyophilized powder was dissolved in water to 4000 U/ml.

Popcorn inhibitor, used as a specific inhibitor of βFXIIa,[10] was purchased from Unicorn Diagnostics Ltd. (London, UK). The inhibitor was reconstituted in distilled water to a concentration of 4 μmol/L, dispensed in 200-μl aliquots and stored at −70°C.

Soybean trypsin inhibitor, type I-S, specific for kallikrein[11] was supplied by the Sigma Chemical Company (St. Louis, MO). A 5-μmol/L solution was prepared in water and stored at −70°C.

Trasylol (aprotinin) was supplied by Bayer (Leverkusen, Germany), and 500,000 KIE were diluted in 50 ml NaCl 0.15 mol/L.

Venous blood from 20 normal healthy donors was drawn into 0.13 mol/L sodium citrate solution (9 vol + 1 vol) and centrifuged at room temperature for 20 min at 3000 × *g* to obtain platelet-poor plasma. Pooled platelet-poor plasma was dialyzed against 50 mmol/L Tris-HCl buffer, pH 7.4, containing 0.1 mol/L NaCl and 1.0 mmol/L Na_2-EDTA to remove trisodium citrate,[12] dispensed in small aliquots, and stored at −70°C.

Human factor XII (FXII)-deficient plasma and human-prokallikrein-deficient plasma were obtained from Helena Laboratories (Beaumont, TX).

Antibodies against human antithrombin and human complement C1 esterase inhibitor, purified immunoglobulin fraction of rabbit antiserum, were purchased from Dako (Glostrup, Denmark).

CNBr-activated Sepharose 4B was purchased from Pharmacia. The antibodies against antithrombin and complement C1 esterase inhibitor were coupled according to the manufacturer's instructions. The substituted gels contained approximately 10 mg/ml of the respective antibodies, measured as absorbance at 280 nm [A_{280}] of the antibody solutions at the beginning and end of the coupling reaction.

Polyethylene tubing (Portex, Hythe, UK) with an inner diameter of 1.0 mm was surface-modified by covalent end-point immobilization of heparin as previously described.[3] Briefly, heparin was partially depolymerized by reaction with nitrous acid, yielding anhydromannose units with free aldehyde functions at the new reducing termini. The partially depolymerized heparin was coupled by reductive amination to free amino groups in polyethylene imine, adsorbed to the plastic surface. The resulting surface density of heparin is about 2 μg/cm^2,[13] with an antithrombin-binding capacity in the range of 18–22 pmol/cm^2. Approximately 5 pmol/cm^2 correspond to high-affinity antithrombin-binding sites, the remainder representing sites with lower affinity for antithrombin.[14] In the following text, this surface is called the *unfractionated heparin surface*.

For the preparation of *low-affinity heparin surfaces*, the partially depolymerized polysaccharide was fractionated on a column of antithrombin–Sepharose to remove all antithrombin-binding heparin. Material that did not bind to the immobilized antithrombin at an NaCl concentration of 0.25 mol/L (in 50 mmol/L Tris-HCl, pH 7.4) was considered to be low-affinity heparin, and was immobilized as described above. The low-affinity heparin surface bound no measurable amounts of antithrombin.

We also used two buffers: 50 mmol/L Tris-HCl, pH 7.4, containing NaCl to a final ionic strength of 0.15, polyethylene glycol (PEG 6000) 0.2%, and human serum albumin (HSA) 0.5%; and for assay buffer, 50 mmol/L Tris-HCl 0.14 mol/L NaCl, pH 7.8, was used in FXII and activated FXII (FXIIa) determinations.

METHODS

Antithrombin was determined by an FXa inhibition assay as described previously.[7] Thrombin–anti-

thrombin complex and complement C1 esterase inhibitor concentrations in plasma were determined using commercial kits.[15,16]

For depletion of antithrombin and complement C1 esterase inhibitor in plasma, 40-ml portions of dialyzed platelet-poor plasma or FXII-deficient plasma were passed at 4°C through 17-ml columns of antibody-substituted gels, either anti-antithrombin Sepharose 4B or anti-complement C1 esterase inhibitor Sepharose 4B, equilibrated in 0.15 mol/L NaCl, pH 7.4. Fractions of 5 ml were collected and assayed for the respective inhibitor, spontaneous proteolytic activity against S-2302[17] and protein concentration [A_{280}]. Fractions containing <0.005 antithrombin U/ml or complement C1 esterase inhibitor below 2% of normal concentration and exhibiting no measurable spontaneous proteolytic activity were pooled and stored at −70°C. The protein content was 80% of that of the native plasma. The antithrombin-depleted plasma had a normal content of complement C1 esterase inhibitor and the complement C1 esterase inhibitor-depleted plasma was normal with regard to antithrombin. Antithrombin-depleted plasma was further depleted of complement C1 esterase inhibitor by passage through the anti-complement C1 esterase inhibitor column as described before, yielding a preparation lacking both inhibitors with a protein content of 70% of that of the original plasma. The antithrombin-depleted plasma and complement C1 esterase inhibitor-depleted plasma were assayed for coagulation factors XII, XI, X, IX, VIII, V, and prothrombin, as well as for α_2-macroglobulin and α_1-antiplasmin by routine clinical methods, and were found to be within the normal range, even when the dilution factor was disregarded.

EXPERIMENTAL PROCEDURE

Prior to use, 60-cm segments of heparinized tubing (vol, 0.47 ml) were extensively rinsed to remove nonspecifically bound heparin and assayed to confirm that no further release of heparin during exposure to plasma occurred.[7,8] Adsorption of FXII from dialyzed plasma and assays for surface-bound activities were performed by incubating the tubing segments as closed loops with 200 μl of plasma or the specified reagents on a rotating slanted table turn for 15 min at a flow velocity of 330 cm/min.

Surface-bound total FXII (FXII + activated FXII) was measured as previously described.[8] In short, following incubation with plasma, the tubing segments were rinsed with 5 ml Tris-buffer and then exposed to a mixture of the FXII activator Cephotest (200 μl), FXII-deficient plasma contributing the FXIIa substrate prokallikrein (10 μl), and assay buffer (20 μl). The reaction mixture was transferred to a test tube and kallikrein activity was immediately quantified by addition of the chromogenic substrate S-2302. The substrate reaction was terminated after 5 min by the addition of citric acid, and the absorbance at 405 nm was read. Selective determination of surface-bound spontaneously activated FXII was performed by replacing Cephotest by assay buffer in the reagent mixture. S-2302 to some extent crossreacts with other plasma proteinases,[18] but because substrate hydrolysis was always abolished by addition of the kallikrein-specific inhibitor soybean trypsin inhibitor to the assay mixture, the absorbance readings were regarded to reflect only kallikrein activity.

The surface-exposed plasma aliquots were immediately assayed for kallikrein activity as follows: 20 μl of the exposed plasma was incubated with 200 μl assay buffer and 200 μl S-2302 at 37°C for 5 min. The reaction was stopped with citric acid and A_{405} was read. The data were corrected for the absorbance of reagent blanks. Nonparametric Mann–Whitney tests were used to compare groups.

RESULTS

Essentially similar quantities of total FXII were taken up by the unfractionated heparin and low-affinity heparin surfaces during exposure to normal plasma, as seen from the kallikrein activities generated in the subsequent enzymatic assay (Fig. 1A). In agreement with previous results,[8] only minimal activity corresponding to spontaneously activated FXII could be detected on the unfractionated heparin surface, whereas virtually all FXII had been converted to an active enzyme on the low-affinity heparin surface. If the heparin surfaces were exposed to plasma deficient in either FXII or prokallikrein instead of normal plasma, no activities above background could be monitored (not shown). The activities studied thus clearly originated from the plasma contact factors.

The uptake and activation of FXII on the unfractionated heparin surface were then studied with plasma preparations depleted of antithrombin or complement C1 esterase inhibitor, or both. Assays were performed with FXII-deficient substrate plasma lacking the same inhibitors. Incubation of the unfractionated heparin surface with antithrombin-depleted plasma showed substantial activation of the surface-associated FXII, as in the results obtained with the low-affinity heparin surface exposed to normal plasma. If the assay was instead carried out with substrate plasma containing antithrombin, no enzymatic activity was observed on the unfractionated heparin surface (Fig. 1B). In the absence of complement C1 esterase inhibitor, the recovery of total surface-bound

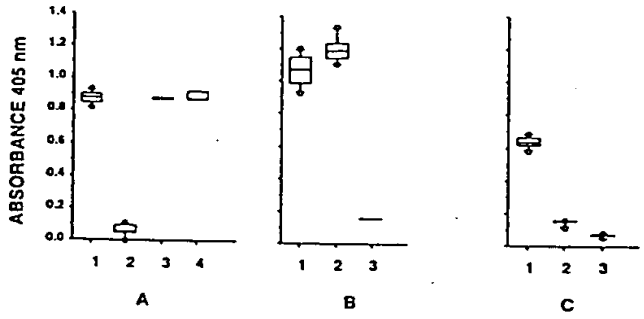

Figure 1. Surface-bound total FXII and enzymatically active FXII on the unfractionated heparin surface and the low-affinity heparin surface following incubation with normal plasma or inhibitor-depleted plasma. The data are presented in box plots (StatView®, Apple). The symbols indicate (beginning from below): circle, lower extreme; horizontal line, 10th percentile; bottom of box, lower quartile; line inside box, median; top of box, upper quartile; horizontal line, 90th percentile; and circle, upper extreme. Each box represents six observations. A: The unfractionated heparin and low-affinity heparin surfaces after exposure to normal plasma. 1: Total FXII on the unfractionated heparin surface; 2: spontaneously activated FXII on the unfractionated heparin surface; 3: total FXII on the low affinity-heparin surface; 4: spontaneously activated FXII on the low-affinity heparin surface. B: The unfractionated heparin surface after exposure to antithrombin-depleted plasma. 1: Total FXII assayed with antithrombin-depleted substrate plasma; 2: spontaneously activated FXII assayed with antithrombin-depleted substrate plasma; 3: spontaneously activated FXII assayed with substrate plasma containing antithrombin. C: The unfractionated heparin surface after exposure to complement C1 esterase inhibitor-depleted plasma. 1: Total FXII assayed with complement C1 esterase inhibitor-depleted substrate plasma; 2: spontaneously activated FXII assayed with complement C1 esterase inhibitor-depleted substrate plasma; 3: spontaneously activated FXII assayed with substrate plasma containing complement C1 esterase inhibitor.

FXII was somewhat lower than in the preceding experiment (Fig. 1C). The fraction of activated FXII was considerably lower than with antithrombin-depleted plasma, but higher than with normal plasma. The presence of complement C1 esterase inhibitor in the substrate plasma quenched this small yield of active FXII ($P < .05$). When both inhibitors had been removed from the experimental system, the outcome was the same as when antithrombin only was lacking (not shown). The results clearly demonstrate that antithrombin and the immobilized functionally active heparin molecules are essential components of the control FXII activation on the unfractionated heparin surface.

The concentration of antithrombin required to suppress spontaneous FXII activation on the unfractionated heparin surface was studied using antithrombin-depleted plasma reconstituted with purified antithrombin to defined levels. The subsequent assay procedure was performed with antithrombin-free substrate plasma. As shown in Figure 2, the recovery of total FXII on the surface after exposure to plasma was largely independent of the antithrombin concentrations. The fraction of spontaneously activated FXII, on the other hand, decreased dramatically with increasing antithrombin concentrations, reaching a steady background level at slightly above 10% of the normal concentration.

Normal plasma was remarkably unaffected by exposure to the unfractionated heparin surface, whereas incubation with the low-affinity heparin surface caused substantial activation (Fig. 3A and B). Plasma lacking either antithrombin or complement C1 esterase inhibitor and, in particular, plasma devoid of both inhibitors were activated in contact with the unfractionated heparin surface. The contribution of activated FXII to these activities was visualized by their varying susceptibilities to popcorn inhibitor, specific for βFXIIa[10] (Fig. 3C–E).

We studied the effect of the two heparin surfaces on the entire plasma coagulation system by circulating aliquots of recalcified plasma (20 μl of 1 mol/L $CaCl_2$ per ml plasma) in the rotating loops. Clotting times were determined and thrombin generation was measured as thrombin-antithrombin complex formation.[12] Normal plasma in contact with the low-affinity heparin surface clotted after about 12 min, and there was substantial thrombin generation in aliquots sampled shortly before clotting. Antithrombin-depleted plasma exposed to the unfractionated heparin surface had a similar clotting time, but for obvious reasons thrombin-antithrombin complex determinations were not meaningful. Normal plasma and complement C1 esterase inhibitor-depleted plasma showed insignificant thrombin activation and no sign of clotting dur-

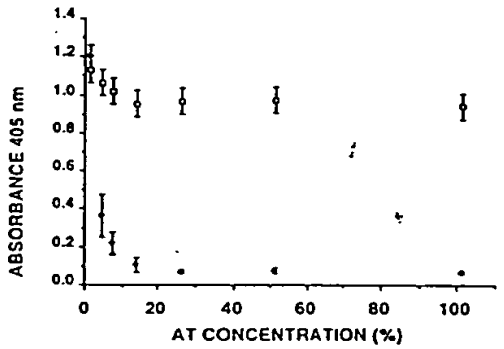

Figure 2. Total FXII and spontaneously activated FXII on the unfractionated heparin surface after exposure to antithrombin-depleted plasma reconstituted with purified antithrombin in different concentrations. Open squares indicate total FXII, solid squares spontaneously activated FXII. Mean and SD, $n = 6$.

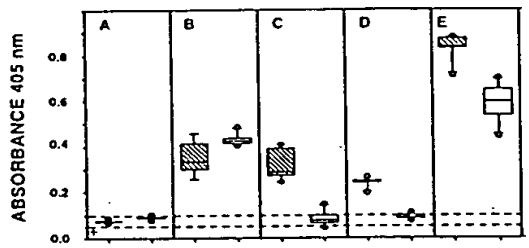

Figure 3. Enzymatic activity in normal plasma and in inhibitor-depleted plasma samples after incubation in contact with the unfractionated heparin surface or the low-affinity heparin surface for 60 min. Measurements were made in the absence (boxes on left) or in the presence of the βFXIIa-specific inhibitor popcorn inhibitor (boxes on right) and the kallikrein inhibitor soybean trypsin inhibitor (see below). Box plot (for symbols, see Fig. 1). A: Normal plasma exposed to the unfractionated heparin surface. B: Normal plasma exposed to the low-affinity heparin surface. C: Antithrombin-depleted plasma exposed to the unfractionated heparin surface. D: Complement C1 esterase inhibitor-depleted plasma exposed to the unfractionated heparin surface. E: Antithrombin- and complement C1 esterase inhibitor-depleted plasma exposed to the unfractionated heparin surface. The hatched horizontal lines represent the 90% confidence intervals for all results obtained in the presence of soybean trypsin inhibitor (n = 24). The concentrations of soybean trypsin inhibitor and popcorn inhibitor were 5 and 4 μmol/L, respectively, corresponding to >100-fold excess over FXII and prokallikrein concentrations in the incubation mixtures.[10,11] +, The activity in plasma analysed prior to surface exposure. The slightly increased absorbance obtained in the presence of soybean trypsin inhibitor, and also after addition of aprotinin (not shown), was regarded as a nonspecifically enhanced background.

ing 60 min exposure to the unfractionated heparin surface (Table I). The plasma samples that never clotted in contact with the heparin surface were transferred to polystyrene tubes and found to have clotting times similar that of the original recalcified plasma. Experiments with the unfractionated heparin surface and antithrombin-depleted plasma reconstituted with increasing amounts of purified antithrombin showed that clotting ceased at about the same antithrombin concentration as did the spontaneous activation of FXII (cf. Fig. 2 and Table I).

DISCUSSION

A common trend in the field of blood compatibility of artificial materials has been to strive for materials that reduce the activation of the defense systems in blood by controlling the composition and structure of plasma proteins adsorbed to the surface, e.g., by selective uptake of albumin. The present approach was instead designed to find out how functionally active heparin immobilized on an artificial surface can actively support the natural control mechanisms in blood to prevent unwanted and uncontrolled activation of the contact system.

Adsorption of the complex of contact activation factors in plasma (FXII, prokallikrein, FXI, and high-molecular-weight kininogen) to foreign surfaces results in an activation process. Initially, surface-adsorbed FXII is converted to enzymatically active αFXIIa, which activates prokallikrein, further FXII, FXI, and, in a calcium-dependent manner, the intrinsic coagulation system.[1] Cleavage of surface adsorbed αFXIIa by kallikrein leads to formation of a fragment, βFXIIa containing the active site, which is released into the liquid phase. βFXIIa activates prokallikrein with concomitant activation of further FXII.[19]

Because of the abundance of sulphate groups, heparin surfaces are negatively charged, as is easily visualized from their wetting properties. Since negatively charged materials in particular are known to activate FXII, it may appear contradictory that activation of FXII was observed on only the low-affinity

TABLE I
Clotting Times and Thrombin Generation Measured as Thrombin-Antithrombin Complex Formation in Recalcified Plasma Exposed to Heparin Surfaces

	Antithrombin	Clotting Time (min)	Thrombin Generation (pmol/ml)
Low-affinity heparin surface	100%	12 ± 2 (n = 6)	39.2 ± 6.9*
Unfractionated heparin surface	0%	12 ± 1.3 (n = 6)	
	1.5% (42 pmol/ml)	14.5 ± 0.5 (n = 6)	ND
	3% (84 pmol/ml)	23.5 ± 1.5 (n = 6)	ND
	6% (170 pmol/ml)	42.0 ± 5.0 (n = 6)	ND
	12% (340 pmol/ml)	>60 (n = 6)	0.17 ± 0.14
	100% (2800 pmol/ml)	>60 (n = 6)	0.11 ± 0.03
	100% C1-INH-depleted plasma	>60 (n = 6)	0.14 ± 0.04

The preparations studied included normal plasma, antithrombin-depleted-plasma reconstituted with purified antithrombin in different concentrations, and complement C1 esterase inhibitor-depleted plasma. The background level of the thrombin–antithrombin complex in the plasma fractions was 0.02–0.03 pmol/ml. ND, Not determined.
*Measurements of plasma recovered from separate loops at 11 min.

heparin surface. The results obtained after removal of antithrombin from plasma, however, showed as extensive FXII activation on the unfractionated heparin surface as on the low-affinity heparin surface at normal inhibitor concentration. Obviously the suppression of FXII activation on the heparin surface is based on the concerted action of two critical factors: antithrombin in plasma and the biologically active heparin molecule on the surface. Moreover, our previous finding that extensive FXII activation occurred on the low-affinity heparin surface even after the addition of heparin to the surface-exposed plasma clearly shows that the heparin molecule must be surface-immobilized to prevent contact activation.[8]

Complement C1 esterase inhibitor has been shown to be a major inhibitor of plasma βFXIIa and kallikrein.[19–21] Indeed, plasma lacking this inhibitor exhibited substantial enzymatic activity after exposure to the unfractionated heparin surface. A similar degree of activation was found in antithrombin-depleted plasma. These plasma activities, however, probably have evolved by different mechanisms. In plasma lacking complement C1 esterase inhibitor, the control of the activation process in the liquid phase would seem to be impaired, allowing the propagation of activity spontaneously generated in plasma or originating from a possible minute activation on the surface. In the absence of antithrombin, there is extensive activation on the surface, and the activity transmitted into the fluid phase may well exceed the inhibitory capacity of complement C1 esterase inhibitor and the other inhibitors in plasma. The high activity in plasma depleted of both inhibitors—i.e., lacking control of activation both on the surface and in the liquid phase—is perfectly in line with this assumption. In addition, complement C1 esterase inhibitor seems to have a minor but measurable effect on the suppression of FXII activation on the unfractionated heparin surface, as seen in Figure 1C.

Despite similar degrees of activation in samples of antithrombin-depleted plasma and complement C1 esterase inhibitor-depleted plasma after exposure to the unfractionated-heparin surface, only the former clotted, confirming a poor activating potential of βFXIIa toward FXI and the intrinsic coagulation system.[19] Hence the triggering of the coagulation cascade in the present experimental system must be linked to the extensive generation of surface associated αFXIIa. This conclusion is supported by the results obtained with the low-affinity heparin surface, and is also in accord with the mechanism suggested by Pixley et al.[21]

The results show that contact activation and clot formation on the unfractionated heparin surface ceased at about the same, surprisingly low antithrombin concentration. Thrombin generation in the unclotted recalcified surface–exposed plasma samples, measured as thrombin–antithrombin complex,[7] was insignificant. Apparently the major inhibitory effect of the unfractionated heparin surface toward the coagulation enzyme system is not directed against thrombin, but rather at a stage preceding prothrombin activation. Antithrombin taken up on the immobilized high-affinity antithrombin-binding heparin sequences mediates rapid inhibition of FXa and thrombin,[6,7] and αFXIIa may well be under the control of a similar mechanism. If so, FXII molecules adsorbed to the unfractionated heparin surface may indeed be converted to αFXIIa, but are instantaneously neutralized by heparin-bound antithrombin, present in an activated form and at a very high local concentration. The subsequent amplification of the contact activation process via kallikrein generation and feedback activation of further FXII would thereby be effectively blocked. It is tempting to suggest that the suppressed FXII activation described in the present report represents a main component not only of the thromboresistant properties of the unfractionated heparin surface but also of its antifibrinolytic properties and possibly its compatibility with the complement system described in experimental as well as in clinical reports.[4,5,22–26] Heparin in plasma has an insignificant effect on activated FXII, although the susceptibility of α- and βFXIIa to heparin–antithrombin has been described in purified systems.[9,27] Obviously, the potent suppression of the activation mechanism for surface-absorbed FXII and of the contact activation process in plasma is a property unique to surface-immobilized, functionally active heparin.

The heparan sulphate of the endothelial lining, with a structure closely related to that of heparin, exposes antithrombin-binding sequences to the circulating blood.[28] The present results obtained with heparin immobilized on an artificial surface thus may illustrate an essential function of the endothelial heparan sulphate for the control of contact activation on the intact vascular wall.

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European Society of Cardiology Conference

Heparin-coated stents could radically cut costs. David Fim reports on this and other developments presented in Amsterdam.

Heparin-coated stents could radically reduce the cost of stenting, paving the way for more widespread use. Hospital stay was cut from 8.5 days to 3.1 as a result of lower complications in a pilot study of Johnson & Johnson Interventional Systems' polyamine-heparin-coated Palmaz-Schatz stent.

Data from the Benestent II pilot study confirm the theory that heparin coatings can eliminate the need for anticoagulation which in turn reduces bleeding complications – one of the biggest problems for stent patients. With lower complications there are fewer transfusions and less surgery: hospital stay can be reduced.

Professor Patrick Serruys, professor of interventional cardiology and director of the catheterisation laboratory at the Thoraxcenter, Rotterdam, presented the pilot data at the annual congress of the European Society of Cardiology (Amsterdam, August 20th-24th).

The Benestent II pilot study comprised three initial phases, where resumption of heparin therapy after sheath removal was postponed progressively by six, 12 and 36 hours. In Phase IV, Coumadin and heparin were replaced by antiplatelet agents, ticlopidine and aspirin. Procedural heparin was administered according to activated coagulation times. Sub-acute thrombosis did not occur in any patient, despite the change in drug regimen. The overall clinical success rate was 99%, compared with 93% in Benestent I.

Three-month event-free survival was 86%, compared with 80% for current stenting practices and 70% for balloon angioplasty.

Prof Serruys said better technique and patient selection also contributed to the improvement in outcome in the pilot study. In particular the greater post-procedural minimum luminal diameter (MLD) is due to routine high pressure balloon inflation.

Six-month restenosis

	MLD (mm)		Restenosis
	Acute	six-months	(%)
Benestent I	2.51	1.85	20
Benestent II (pilot I-IV combined)	2.77	2.08	13
	Gain (mm)	Loss (mm)	Restenosis (%)
Phase I	1.67	0.65	15
Phase II	1.67	0.94	20
Phase III	1.67	0.55	10
Phase IV	1.68	0.58	6

. . . outcomes

Data from Benestent I put the cost of stenting including subsequent complications at about Fl 20,000 ($32,000) compared with Fl 10,000 for PTCA, said Dr Ben van Hout of the Institute for Medical Technology Assessment at Erasmus University, Rotterdam. The decrease in ischaemic events during the first year contributed a saving of about Fl 1,500, which brings the additional cost of stenting to about Fl 8,500. The increase in event-free survival is about 11% in the first year, which represents an additional cost of Fl 77,500 per event-free survivor: "Much higher than the corresponding costs per event-free survivor following PTCA," said Dr van Hout.

However, the data are based on very limited information. They are drawn from *ex ante* analysis, without direct data, indirect costs or information about healthcare outside the confines of the trial. No account has been taken of quality of life or the additional costs to society, such as absence from work. Most notably the results do not take account of advancing stent technology.

Dr van Hout is in charge of the outcomes arm of Benestent II and he promises it will be one of the least biased studies yet performed. The protocol includes direct gathering of resource use, indirect costs and quality of life, in addition to patient passports, to track costs outside the trial.

MUST validates ticlopidine use

Antiplatelet drugs cut thrombosis and cause very few complications, according to Dr Marie Claude Morice of the Institut Cardiovasculaire Paris Sud, France. Presenting preliminary data from the MUST trial, she said 260 stent patients treated with aspirin and ticlopidine had a sub-acute occlusion rate of just 1.15%. CABG was needed in only 0.38% of cases and MI occurred in only 1.92%. There were no vascular complications and only one case of gastro-intestinal bleeding. Most patients were discharged within two days. Dr Morice's group has used antiplatelet therapy with reduced heparin in over 1,150 patients, with sub-acute thrombosis in the region of 1.2-1.6%.

ACCESS highlights transradial PTCA problems

Procedural outcomes are the same for radial, brachial and femoral artery PTCA but entry failure is more common during transradial procedures. Bleeding complications are more likely following transbrachial access, said Dr Ferdinand Kiemeneij, of the Amsterdam Department of Interventional Cardiology during a presentation of data from the ACCESS study.

The ACCESS group has randomised 900 elective PTCA patients to either transradial (TRA), transfemoral (TFA) or transbrachial (TBA) PTCA and interim results from 450 were presented.

	TRA	TBA	TFA
Access failure (%)	7.9	3.4	0.4
Major bleeding (%)	1.0	4.3	1.3
PTCA success (%)	93.4	95.1	96.0
Cardiac events (%)	7.2	8.4	7.1
Procedure time (min)	37	33	30
Fluoroscopy time (min)	9.6	8.9	8.9

STUDY SUPPORTS OUTPATIENT PTCA

A sub-group of ACCESS patients triaged to outpatient status after coronary stenting suffered no major cardiac or vascular compli - cations.

None of the 100 stent implant patients actually left hospital for safety and legal reasons, but the favourable outcome for the transradial PTCA group in general as well as the 45 patients identified as safe-to-discharge form the basis for true outpatient angioplasty, said Dr Kiemeneij.

RADIAL ROUTE SUFFERS FROM OCCLUSION

Miniaturisation of angioplasty equipment has rekindled enthusiasm for radial artery catheterisation but it should not be used for coronary angiography because of the high rate of delayed occlusion. Procedural complications during transradial catheterisation are rare. However the late occlusion rate is over 10%, Dr Laurent Quillet of Trousseau University Hospital, Tours, France, told delegates at a session on technical improvements in PTCA.

Transradial catheterisation reduces the risk of local haematoma, especially in the case of full heparinisation; allows more rapid mobilisation of patients and earlier hospital discharge; reduces pain; and cuts hospital costs. The main drawback of the transradial approach is that it limits the maximum size of device, which excludes the Rotablator and the Viktor and Roubin stents.

Dr Quillet presented data from a 201 patient risk/benefit analysis of left-radial catheterisation. Overall success for angiography, balloon angioplasty and stenting was 98%. Radial patency for the first 106 patients was 88.7% at four months. Occlusion was not related to anticoagulation or gender.

The left radial artery is slightly more awkward than the right from the physician's point of view, but most patients are right handed and the subclavian artery is a safer route than the innominate artery, said Dr Quillet. Conventional catheters can be used in either the radial or femoral artery.

PRIMARY ANGIOPLASTY BEATS STREPTOKINASE AT THREE YEARS

MI patients treated by primary angioplasty are more likely to survive three years, have less re-infarctions and better ventricular function than those treated with streptokinase. Data from 301 patients in the Zwolle trial (Weezenlander Hospital, Zwolle, the Netherlands) shows that three-year mortality for primary angioplasty is 2.5% compared with 6.4% in the streptokinase group. In order to salvage viable myocardium, it is necessary to restore normal bloodflow rapidly and completely. However, thrombolysis fails to restore flow in 20% of patients and as many as half do not achieve full restoration of flow. Re-occlusion occurs in 15-30% of patients.

Dr Aylee Liem, a cardiologist at the Weezenlander Hospital, said that normal bloodflow was restored in 97% of PTCA-treated patients and that the size of the infarct, calculated from enzyme data, was smaller in the PTCA group. Average follow-up was 31 months.

There was no difference in medical costs between the two treatments. Higher initial PTCA costs were offset by lower readmissions. However, Dr Liem cautioned that the study was undertaken at an experienced centre with 24-hour angioplasty facilities and on-site surgical back-up.

UK TEAM USES MINI-MI TO CURE OBSTRUCTIVE CARDIOMYOPATHY

A team of cardiologists from the Royal Brompton Hospital in London has used a balloon technique to treat hypertrophic obstructive cardiomyopathy. The technique, developed by consultant cardiologist Professor Ulrich Sigwart, uses a balloon to induce ischaemia in the part of the heart that is causing the obstruction, usually a region of the anterior wall.

"We infarct the bulge," said honorary senior registrar Dr Charles Knight, who presented the technique at the congress. When the relevant region has been identified, pure alcohol is passed down the catheter to kill the muscle permanently.

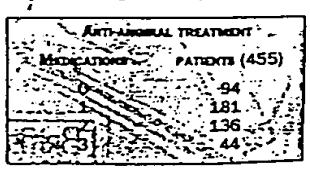
Obstructive hypertrophic cardiomyopathy causes exuberant muscle growth which can impede ejection from the left ventricle. It tends to affect young people and can be fatal. Conventional treatment uses pacing to make the heart less vigorous or surgery to remove the obstructive myocardium. Pacemakers need to be replaced every ten years and surgery carries a 5% mortality risk. Six patients aged 14-18 have been treated so far, and all are doing well, said Dr Knight. The balloon treatment requires only a two-day hospital stay, compared with a week to ten days for heart surgery.

TRANSGENIC COAT MAY PREVENT STENT RESTENOSIS

Israeli researchers say they have successfully coated stents with genetically engineered endothelial cells. Researchers at the Lady Davis Carmel Medical Centre in Haifa, Israel, have succeeded in harvesting, transforming and culturing human cells and are currently testing coated stents in animals. They hope the transgenic cells will produce antithrombotic agents until the endothelium of the coronary artery engulfs the stent; a process which normally takes several weeks. Dr Moshe Flugelman says the group hopes to start human clinical trials in the next 12 months.

TEN-YEAR FOLLOW UP SHOWS HIGH SURVIVAL FOR PTCA

As many as 85% of PTCA patients survive at least ten years and almost half experience no subsequent cardiac events. A German study of 555 patients who underwent single vessel PTCA between 1983 and 1985 found that 11% had suffered an MI, 20% needed bypass surgery and 26% redilatation. Only 15% died. Event-free survival was 47%, while 60% of patients were asymptomatic and 20% no longer needed anti-anginal drugs – a measure of clinical restenosis. Most cardiac events occurred during the first year.



More news from the ESC conference next week

Intermedics' Res Q gets US panel go-ahead

Intermedics' (US) Res Q arrhythmia control device was unanimously recommended for approval by the US FDA's Circulatory System Devices Advisory Panel on August 21st. The implantable device is intended for patients who are at high risk of cardiac arrest as determined by a previous episode of loss of consciousness due to a ventricular arrhythmia.

The PMA included data from two prospective non-randomised trials that used historical controls from a trial of an analogous device by Medtronic (US). The first of Intermedics' trials – the Patch Study – enrolled 178 patients who were followed for a mean of 24 months after the device was implanted. The second trial – the NTL Study – enrolled 235 patients who were followed for a mean of 17 months after implantation. The device was modified after the trials had started and only a total of 268 patients received the actual device reviewed by the panel.

Apart from different leads, the study designs were similar. The results were also similar: patient survival from sudden cardiac death was over 98% and one-year survival of all patients was over 90% in both studies. These results were considered acceptable when compared with historical data.

Among issues considered by panel members was the activation of several devices when the patients passed through magnetic security monitors in stores. Intermedics is working on this issue. Although patients need to return to the physician's office every four months to have the device's capacitor serviced, they are not expected to be put at unacceptable risk if they miss the appointment.

St Jude gets clearance for pacemaker lead trials

St Jude Medical has been granted US FDA clearance to begin clinical trials of its Tendril DX active fixation leads. Developed by St Jude's cardiac rhythm business, Pacesetter, the lead contains a steroid matrix in its helix tip which is released once the implanted lead comes into contact with body fluid. The steroid is intended to suppress the body's inflammatory response to a foreign substance.

The lead's electrode tip is coated with titanium nitride, which reduces polarisation and increases sensing characteristics, says St Jude. "The smooth active fixation mechanism is designed to reduce the dislodgements more frequently associated with passive fixation leads thus resulting in the potential for earlier patient discharges," says Pacesetter's president, Eric Sivertson. Tendril DX leads are available in five lengths and can be placed in either the atrium or the ventricle.

Guidant initiates US stent trial

Guidant's Advanced Cardiovascular Systems division has initiated human clinical trials of its Multi-Link stent. The 15 mm device is constructed from linked rings and is mounted on a delivery system that incorporates a selection of low-profile balloons. An elastomer membrane is used to transfer inflation force evenly from the balloon to the stent.

The US implants, which took place at the end of August, are part of an initial study that will be followed by a randomised multicentre trial. Clinical trials in Europe and Japan began last year. WEST – the Western Europe Stent Trial – will eventually include 100 patients at seven centres while the Japanese study will include 60 patients at three sites.

IN BRIEF

❑ ***First implants for Guidant/CPI's Ventak Mini***

The first implants of Guidant subsidiary Cardiac Pacemakers' Ventak Mini family of automatic implantable cardioverter defibrillators have been carried out in Europe. The company claims that, at 68 cm^3 and weighing 125 g, the device is the world's smallest full-featured implantable defibrillator. The Mini is 30% smaller than CPI's most recently released Ventak PRx III. The system includes the Ventak Mini pulse generator, the Endotak DSP defibrillation lead and the model 2950 programmer-recorder-monitor. It also incorporates CPI's optimal biphasic waveform for lowering the energy requirements for heart rhythm correction, and six minutes of stored electrograms to assist in programming and follow-up.

❑ ***New pacemaker monitor from Instromedix***

Instromedix (US) has received US FDA clearance to market its CarryAll pacemaker monitor. The portable unit enables patients to relay information about their implanted devices from home to their healthcare facility. It is compatible with single chamber, dual chamber and rate responsive pacemakers in unipolar and bipolar pacing modes. Oregon-based Instromedix says the CarryAll's algorithms enable better discrimination between pacing and non-pacing pulses as well as improved visualisation of heart electrical activity and pacing ability to stimulate the heart.